UNIVERSITA' DEGLI STUDI DI PADOVA

Dipartimento di Scienze Chimiche

Corso di laurea magistrale in Chimica

TESI DI LAUREA MAGISTRALE

Study of the Cellulose-peptide Conjugation for the Development of Antimicrobial Cotton Textiles

Relatrice: Prof. Peggion Cristina

Controrelatore: Prof. Casalini Stefano

LAUREANDA: Albini Francesca

ANNO ACCADEMICO 2021/2022

ABSTRACT

The aim of this thesis is the study of chemoselective ligations in the context of antimicrobial tissues.

Cotton is the chosen textile because it is a natural fiber, widely produced, air permeable and easy to functionalize. The selected class of antimicrobial agents are antimicrobial peptides. In fact, they present a broad-spectrum mechanism of action. Thus, they are at a lower risk for the resistance development; they are produced by the immune system of different organisms, thus inherently biocompatible and biodegradable.

Thiazolidine and oxime bonds are the selected ligation methods. They are formed by the reaction of an aldehyde and a β-amino thiol (Cysteine) and an alkoxyamine (Aminooxyacetic acid), respectively. The use of a covalent bond is due to the requirement to produce a functionalized textile that is durable, washable and reusable.

The aldehyde is introduced at the C_6 atom of the glucose unit of cotton cellulose by an enzymatic oxidation in a 2,2,6,6-Tetramethyl-1-piperidinyloxyical(TEMPO)/laccase/O_2 system, whereas the Cysteine (Cys) and Aminooxyacetic acid (Aox) are introduced during the peptide synthesis at the N-terminal. Both the oxidizing and the conjugation reactions are interesting for the mild conditions at which they take place.

All the peptides are synthetized through solid phase peptide synthesis (SPPS). Two model peptides are used for the study of the two ligation types. They both have a UV-vis active side chain, a detectable side chain and either a Cys or an Aox. The first is needed to follow the course of the reaction, the second allows to confirm the presence of the peptide on cotton by colorimetric tests and the last two are necessary for the effective ligation with the oxidized cotton. FT-IR analysis is performed to further confirm the presence of the peptides on cotton. The main goals of using these peptides are: the quantification of the peptide loading on different types of cotton, the rough estimation of the aldehyde content on cotton upon the re-use of the oxidizing solution and the evaluation the stability of the two bonds in different media.

Finally, a short analogue of PMAP-36, an antimicrobial peptide, is linked to cotton using the above-mentioned strategy and the antimicrobic activity of the obtained material is tested.

INDEX

1. ABBREVIATIONS

AA	Amino acid
Ac	Acetate
ACN	Acetonitrile
AcOH	Acetic acid
Al_2O_3	Aluminum oxide
Ala (A)	Alanine
AMPs	Antimicrobial peptides
Aox (A_x)	Aminooxyacetic acid
Arg (R)	Arginine
Asn (N)	Aspargine
Boc	Tert-butyloxycarbonyl
Bzl	Benzoyl
Cys (C)	Cysteine
DCM	Dichloromethane
Dde	2-Acetyldimedone
DIC	N,N′-Diisopropylcarbodiimide
DIPEA	N,N-diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DODT	3,6-Dioxa-1,8-octanedithiol
Et_2O	Diethyl ether
EtOH	Ethanol
Fmoc	9-Fluorenylmethoxycarbonyl
Gly (G)	Glycine
H_2O_2	Hydrogen peroxide
HBTU	N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HCl	Hydrogen chloride
HDP	Host defense peptides
HF	Hydrofluoric acid
His (H)	Histidine
HOBt	1-hydroxy-1H-benzotriazole
Ile (I)	Isoleucine
KBr	Potassium bromide
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
Leu (L)	Leucine
Lys (K)	Lysine
MeOH	Methanol
Na_2CO_3	Sodium carbonate
NaBr	Sodium bromide
NaCl	Sodium chloride
NaClO	Sodium hypochlorite

NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NaOAc	Sodium acetate
$NaOCl_2$	Sodium chlorite
NaOH	Sodium hydroxide
NCL	Native chemical ligation
Oxyma	Ethyl 2cyano-2-(hydroxyimino) acetate
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl chloride
PBS	Phosphate buffered saline
PCW	Primary cell wall
PG	Permanent protecting group
Phe (F)	Phenylalanine
Pro (P)	Proline
SCW	Secondary cell wall
SeH	Hydrogen selenide
Ser (S)	Serine
SPPS	Solid phase peptide synthesis
tBu	Tert-butyl
TCEP	Tris(2-carboxyethyl)phosphine
TEMPO	2,2,6,6-Tetramethyl-1-piperidinyloxyical
TFA	Trifluoroacetic acid
Thr (T)	Threonine
TIS	Triisopropylsilane
TMS	Tetramethylsilane
TPG	Temporary protecting group
Trp (W)	Tryptophane
Trt	Triphenylmethyl
Val (V)	Valine
VA-044	2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride

2. INTRODUCTION

In hospitals and health care systems the growth of bacteria on tissues such as bandages, gauzes, sheets, coats and surgical scrubs is an important and growing concern worldwide.[1] The unique set of properties of cotton makes it a highly desirable tissue that possess over 50% of the market share among fabric products employed in the textile industry.[2] In fact, it is a natural fiber purchasable at a low cost that possess softness, strength, elasticity, biodegradability, water affinity, air permeability and is comfortable to wear.[2, 3] The only disadvantage of cotton is that its hydrophilic properties favor the growth of microorganisms on it more than synthetic fabrics.[3] This phenomenon not only enhances the transmission of this bacteria, and especially multidrug-resistant ones[1], but may also result in unpleasant odors, color degradation, allergic responses and deterioration of the textile.[2] Therefore, the development of bio-functionalized materials is much needed and in recent years, intensive research has been conducted to maximize antimicrobial properties of cotton.[2]
In this regard several strategies have been used: electron beam grafting of quaternary ammonium compounds on the cotton surface; immobilization of industrial enzymes within the matrix; complexation of metal salts (ex. $AgNO_3$, $ZnSO_4$ and $Zr(SO_4)_2$)[4]; encapsulation of pyrazole-based compounds into liposomal chitosan for textile finishing; and incorporation of nanoparticles of zinc oxide, titanium oxide, copper oxide and silver oxide and silver.[3] Besides the antimicrobial effect of the textile, another important aspect to be taken in consideration is the durability of the material upon common use (mechanical stress, washing at high temperatures, ironing etc.).[2] Of all the cited methods, only inorganic nanoparticles have been proven to possess durability of the antimicrobial properties at some degree; the best reported method that allows it is the use of polymer binders for the immobilization of nanoparticles.[2] A significant downside of Ag nanoparticles is the potential environmental impact of these substances when released both by washing and when they are disposed.[5]

An interesting alternative is the direct use of bio-biopolymers such as the antimicrobial peptides (AMPs) as the antimicrobial agent on cotton.[6, 7] They are intrinsically biodegradable and their broad-spectrum mechanism of action suggests a lower risk of resistance development.[8] Furthermore they could be covalently bound to cotton and, thus, probably much more durable than other methods of functionalization. One interesting possibility is the use of a thiazolidine bond, which has already been proven effective in the ligation of short antimicrobial peptides[6, 7]. This thesis has two main aims. The first is to expand the knowledge of the peptide-cotton thiazolidine bond in the context of peptide-cotton ligation. Furthermore and considering the wide use of the oxime bond in bio-conjugation[9], the second aim is to simultaneously test this last type of bond for the peptide-cotton ligation. This is done to evaluate if the oxime ligation could be better alternative than the thiazolidine one.

2.1 COTTON

Source and Processing of Cotton

Cotton plants belong to the family *Malvaceae*, the tribe *Gossypieae*, and the genus *Gossypium*.[10] Of the thirty-three species currently recognized, only four have a real commercial value: *hirsutum, barbadense, aboreum,* and *herbaceum*. The first two originates from USA and South America, respectively, and constitutes over 90% of the world cotton production. The last two species are known as "Desi", come from Asia and produce shorter and coarser fibers than the American ones.[11] A cotton fiber is the dried cell walls of an elongated (to >2.5 cm in *hirsutum*), thickened and formerly living cell. It derives from the epidermis of the cottonseed and constitutes the seed hairs (trichomes).[10, 12]

Fig 1: Cotton plant before the harvest

The cotton fiber develops its outer perimeter first and then grows inward toward the center of the fiber. In particular, it is divided, from the outside to the inside, in (Fig. 2):[10] [11]

- **Cuticle**: a thin layer composed of waxes and soluble sugars. The first ones are a mixture of waxy alkanes, fatty acids, fatty alcohols and plant steroids. The seconds derives both from plants and insects. The waxes act as a protective barrier both to water penetration and to microbial degradation of the underlying polysaccharides.
- The **primary cell wall** (PCW) that is further divided into two layers. The outer one consists of pectin derivates mainly in the form of pectic acid and rhamnose. The inner layer consists in hemicellulose, typically in the form of xyloglucan and cellulose. The PCW is also the location of proteins, peptides, free amino acids, organic salts and inorganic anions that are essential for the growth of the cotton plant.
- **Winding layer**: the first layer of the secondary cell wall.
- The **secondary cell wall** (SCW) is composed mostly of highly structurally organized cellulose (see below).
- **Lumen**: an empty canal that runs the length of the fiber.

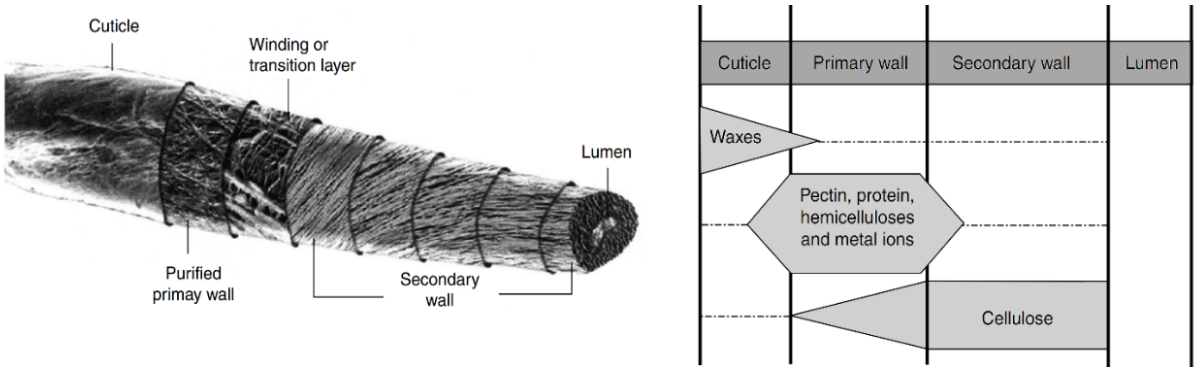


*Fig 2: **On the left**: schematic representation of the structures of a cotton fiber. From the outside to the inside: cuticle, primary wall, winding layer, secondary wall and lumen. **On the right**: distribution of the different components of a cellulose fiber into its structures. Waxes are predominantly present in the cuticle. Pectins, proteins, hemicellulose and metal ions are the major components of the primary wall. Cellulose is mostly present in the secondary wall [13]*

Overall, a mature cotton fiber is composed of 88.0–96.5% cellulose, 1.0–1.9% proteins, 0.4–1.2% waxes, 0.4–1.2% pectins, 0.7–1.6% inorganics and 0.5-8.0% other substances.[10] The variations in the percentage of the components arises from differences in the fiber maturity, variety of cotton and environmental growth conditions.[11]

After harvesting, ginning, mechanical cleaning and spinning the cotton is approximately composed of 95% of cellulose and is usually further subjected to a series of procedures. First of all, the non-cellulosic components are removed through the scouring process to expose the hydroxyl groups of cellulose. This is done with a mixture of wetting agent, NaOH and acetic acid. Then the bleaching process undergoes, usually at boiling temperature with a bath of wetting agent, NaOH, sodium silicate H_2O_2 and acetic acid. At the end the mercerization with 1% boiling NaOH proceeds. Besides the removal of the remaining non-cellulosic components, this process has many beneficial effects, some of them are: improved affinity for dyes, higher water absorption and increased softness.[10] Therefore, the final obtained textile has a cellulose content of over 99%.[11]

Cellulose: Structure and Reactivity

As previously described, cellulose is the major component of cotton. Furthermore, even if it is produced also by other plants and bacteria strains, cotton cellulose is the purest form of natural cellulose and has the highest molecular weight and structural order.[14] Its molecular formula, $C_6H_{10}O_5$, was first discovered in 1838 by Anselme Payen. And the explanation of its polymeric structure is attributed to Hermann Saudinger with a work dated 1920 where he recognized that in cellulose the glucose molecules are covalently linked and not merely aggregated.[15-17]

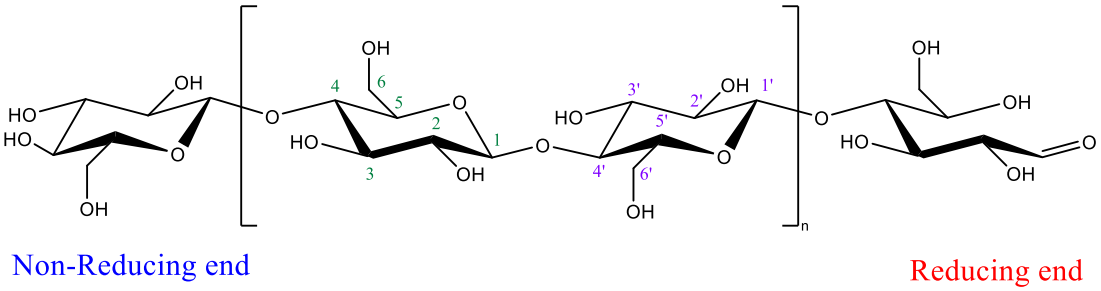


Fig 3: Schematic representation of a cellulose chain. In the center the cellobiose repeating unit. On the left is the non-reducing C_4-OH end and on the right the reducing C_1-OH end.

In particular, cellulose is a linear carbohydrate polymer made of β-D-glucopyranose (or glucose) molecules that are linked to one another by glycosidic bonds between the hydroxyl group of a C_4 of one molecule and the C_1 of an adjacent unit (Fig. 3). Each glucose molecule is rotated 180° with respect to the previous, making the union of two glucose molecules (cellobiose) the repeating unit of the polymer.[10] The hydroxylic moieties at the end a of cellulose chain have different properties: the C_1-OH end is in equilibrium with its aldehyde form and thus presents reducing properties. On the other hand, The C_4-OH of the hemiacetal at the other end (non-reducing end) cannot do so and therefore does not have reducing properties.[17] Each glucose unit has three hydroxyl groups: the C_2-OH and the C_3-OH are secondary alcohols, whereas the C_6-OH is primary alcohol.[14] The presence of hydroxyl group in the chain in abundance permits the creation of both intra- and inter-molecular hydrogen bonds. The firsts give rigidity and strength to the chain, whereas the second ones are needed to form higher ordered aggregates.[10, 17] Specifically, thirty-six cellulosic chains compose an elementary fibril, which in turn is arranged to form microfibrils, which aggregates to microfibril bundles, whose union forms the cellulose fiber (Fig. 4). [10, 18]

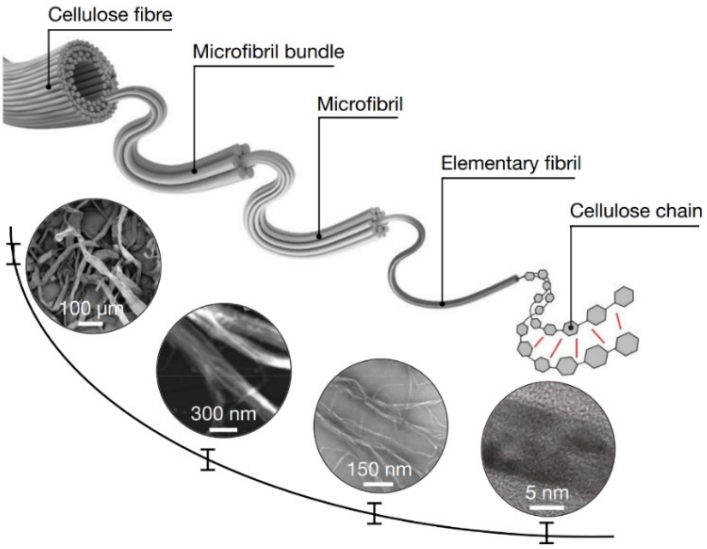


Fig 4: Schematic representation of a cellulose fiber. The length of each unit is noted.[18]

In the cotton, the degree of polymerization (intended as number of glucose molecules per polymer) of cellulose is much larger in the SCW than the PCW. Almost 70-80% of the cellulose in cotton is in the crystalline form I_β, in which the microfibrils are parallel. These crystalline regions are responsible for the strength of the fiber. On the other hand the amorphous regions are essential for elasticity and water or dyes absorption.[10] Interestingly, upon mercerization cellulose changes its crystalline form and becomes a cellulose II, in which the fibers are anti-parallel. Also the size of the crystallite is diminished. [10, 17, 19]

The reactivity of cellulose depends both on the C_2, C_3 and C_6 hydroxyls groups and its supramolecular arrangement: the C_6-OH and the amorphous regions are in general more reactive if compared to the other hydroxyl moieties and the crystalline domains, respectively. Therefore, given the high crystallinity, cotton cellulose has the lowest reactivity of all cellulose sources. Another downside of this material is the low solubility in most common solvents that forces most reaction to be performed heterogeneously and prevents full access hydroxyl groups. Anyhow, the reaction that are most commonly performed on the internal hydroxyls are substitution (esterification or etherification), deoxyhalogentation (to C-Cl, C-Br or acetylation) and oxidation, whereas the terminal aldehyde can be reduced to the hemiacetal or oxidized to a carboxyl. These chemical modifications do not affect drastically the physical-chemical properties of cellulose and makes cellulose more reactive towards possible functionalization agents.[14]

Enzymatic Oxidation of Cotton

The oxidation of the primary alcohol is well known in the literature and the common procedure involves the use of periodate as the oxidizing agent. However in the case of cellulose this reaction is selective toward the C_2-OH and C_3-OH, thus breaking the C-C bond between the two atoms and undermining the integrity of the cellulose chain.[20] In 1983 a stable and water-soluble organic nitrosyl radical called 2,2,6,6-tetramethyl-1-piperidinyloxyical (TEMPO) was introduced for the selective oxidation of primary alcohols to aldehydes in presence of secondary alcohols.[21] This catalyst was proven to be highly effective in the conversion of high molecular weight polysaccharides and ensured high reaction rates, yields, selectivity and modest degradation of the polysaccharide. The drawback of this molecule are the agents that are commonly used for its regeneration: the NaBr/NaClO or NaClO/$NaOCl_2$. The first couple still present the issue of de-polymerization: the alkaline conditions favor the β-elimination at the C_5 that breaks the cellulose chain; also, the hypochlorite is responsible for the C_2-C_3 bond breaking through carbonyl group formation and consequent β-alkoxy fragmentation. The second system diminishes the de-polymerization caused by the hypochlorite but needs to take places at higher temperature and for longer reaction times[20]. Anyhow both of them imply the use of halogenated-based reagents that rise concerns about environmental and safety concerns[20, 22]. Therefore, the use of enzymes was taken in consideration.

An interesting group of enzymes are laccases, given the wide variety of substrates that they can handle. They were first described in 1883, making them one of the oldest enzymes ever described.[23] They belong to a larger

family of enzymes named multicopper oxidase enzymes and can be found both in fungi and higher plants. Their catalytic site is composed of four copper atoms in three redox sites (T1, T2 and T3). The oxidation of the substrate takes places at T1, whereas the T3 copper couple and the T2 copper are 12 Å away from T1 and responsible for the simultaneous reduction of molecular oxygen to water.[24] Although the catalytic site seems to be very conserved, the rest of the molecule shows a wide variability. Specifically, laccases catalyze the oxidation of any substrate similar to the *p*-diphenol.[25] But, in the presence of small molecules, known as redox mediators, they can improve their substrate specificity. For example, molecules that are able to act as electron carriers, remove the steric issues of the direct interaction with the enzyme and permit the oxidation of high molecular weight biopolymers (Fig. 5). [24]

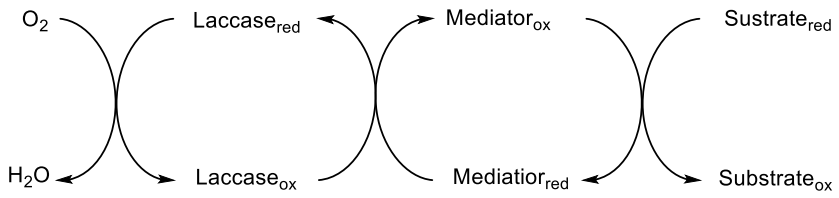


Fig 5: Schematic representation of the generic Laccase-Mediator oxidation.

In this context, in a TEMPO/laccase/O_2 system, TEMPO is the mediator and molecular oxygen the primary oxidant. The ability of laccase to regenerate TEMPO was first investigated by Vikari *et al.* in 2000.[26] The mechanism of action is not yet fully disclosed but the major part was illustrated by Tromp *et al.* in 2010 (Fig. 6).[27] And Aracri *et al.* were the first to use the TEMPO/laccase/O_2 system on cellulose fibers.[28]

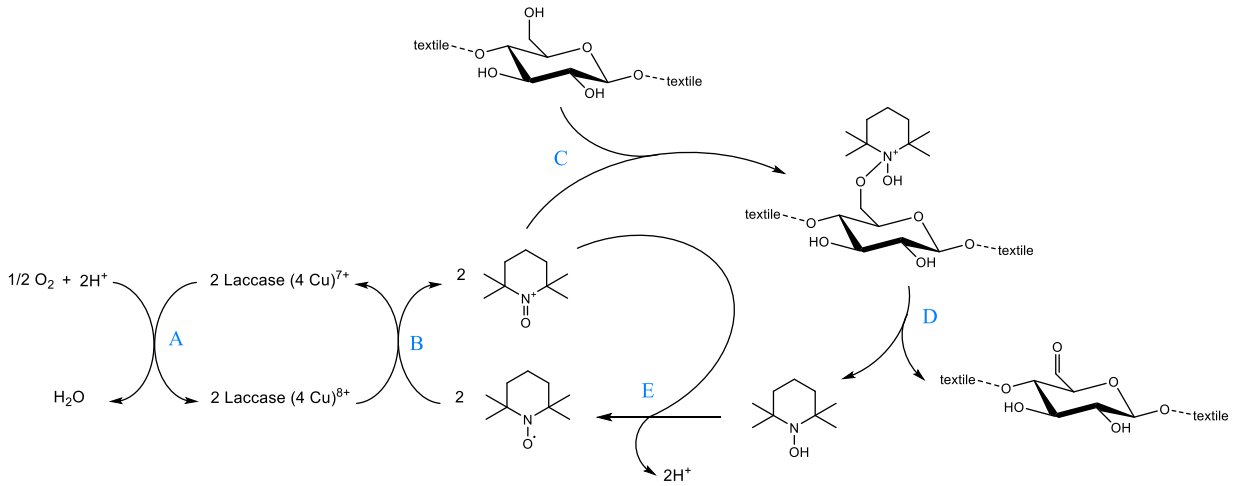


*Fig 6: Overall mechanism TEMPO/laccase/O_2 system. It is important to notice that copper charges conform to the reaction stoichiometry but do not represent the real laccase oxidation state. (**A**) O_2 reduction (**B**) Regeneration of reduced Laccase (**C**) TEMPO chemoselective linkage to the C_6 primary alcohol of cellulose (**D**) Cellulose oxidation (**E**) Compropornation of TEMP [27]*

In the native laccase the fully reduced state is composed of all the copper centers in the +1-oxidation state. The interaction with the O_2 determines the oxidation of one T3 and the T2 coppers. Then the O_2 bond is reduced, all the coppers are oxidized and two molecules of water are produced.[29] Therefore, the oxygen present in the air causes the laccase to be in its oxidized state.

TEMPO (Fig. 6-C and D) binds chemoselectively to the C_6 primary alcohol of cellulose and oxidize it to aldehyde or carboxyl moiety.[27] Aracri *et al.* demonstrated that the higher the TEMPO percentile and the reaction time, the higher is the aldehyde content.[28, 30]

The radical form of TEMPO is regenerated by a comproportionation reaction with TEMPO+ (Fig. 6-E). At the end, laccase regenerates TEMPO+ with its reduction (Fig. 6-B).[22, 27] It was speculated that step-E could be catalyzed by laccase, but there is yet no evidence of it. The rate limiting step in the case of cellulose was demonstrated to be the cellulose oxidation (Fig. 6-D) and the ideal pH is 4-5.[22]

However, it is important to notice that the oxoammonium salts can also deactivate the laccase enzyme by oxidation of the amino acid alcohols. Especially when the alcohol substrate is absent. In fact, the alcohol substrate act as scavengers and prevent the degradation of the enzyme.[31] The enzyme stability can be improved by immobilization, for example by cross-linking.[32]

In conclusion, the TEMPO/laccase/O_2 system is definitely a more ecological and efficient process than the previous herein reported. This is because:

- It does take place at a pH near neutrality and not in an alkali media, thus the β-elimination is disfavored.[28]
- It happens at room temperature and in ca. 20 hours [28].
- The only byproduct is water.
- Does not involve dangerous reactants.

Schiff Test

The Schiff's reagent is a colorimetric and qualitative test that to test the presence of aldehydes. It consists in a solution of pararosaniline hydrochloride and sodium sulfite. The solution is colorless but when added to an aldehyde solution it becomes pink-violet due to the formation of the following adduct:[33]

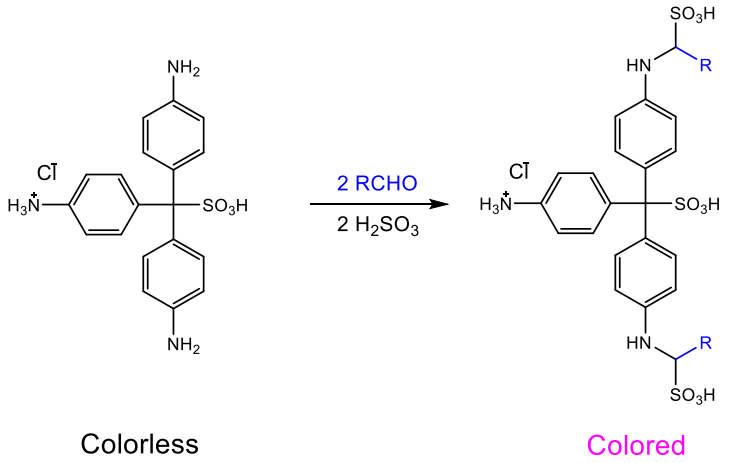


Fig 7: Reaction of the Schiff's reagent with aldehydes. ***On the left*** *the Schiff's reagent;* ***On the right*** *the aminosulfonic acid adduct.*

2.2 CHEMOSELECTIVE LIGATION

Until 30 years ago the chemical synthesis of macropeptides and proteins was stalled. In fact, the innovative and solid phase peptide synthesis (SPPS) could be used efficiently only for the synthesis of a sequence up to ca. 50 amino acids (see paragraph 2.3).[34] But in 1992, Kent *et al.* introduced a new approach to the problem. In fact, they managed to synthetize one of the two subunits of the HIV-1 protease (a 99 amino acidic chain) thanks to a thioester bond between the two segments of amino acidic chain.[35] The principle behind this discovery is the fact that, in the presence of unique and mutually reactive functional groups, two peptides react chemoselectively and form a longer amino acidic chain without the need of protecting groups.[34] (Fig. 8).

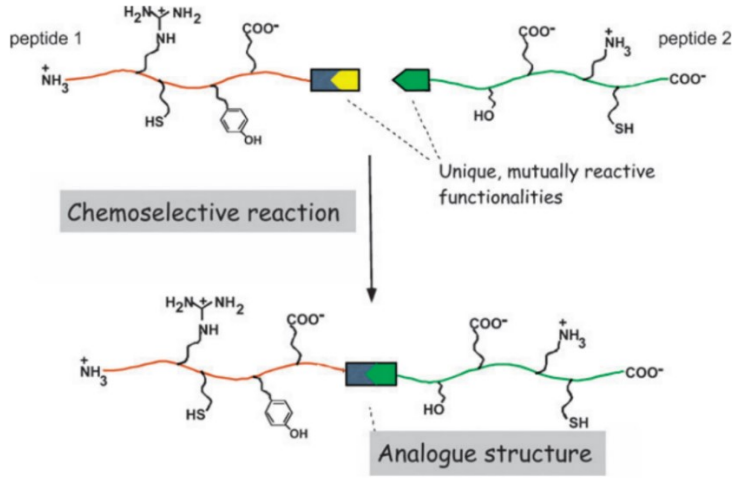


Fig 8: Schematic representation of the principle of the chemoselective ligation. Two molecules react chemoselectively due to the presence of unique and mutually reactive species on both molecules. No protecting groups needed[34]

In this way, the limited solubility and purification issues, characteristic of a standard SPPS of large molecules, are overcame.[34]

The unique and mutually reactive functional groups can be naturally found within the protein or be chemically introduced and thus either create a native bond or not. The reaction between these groups is referred to as chemoselective ligation or click chemistry[36] and nowadays it is not limited to the synthesis of proteins but, for example, it is also exploited to introduce new structural features into the protein structure, or to functionalize biomaterials.[37]

Native Chemical Ligation

The so called "native chemical ligation" (NCL) was first introduced by Dawson *et al.* in 1994.[38] In fact, the reaction between a unprotected synthetic peptide with a C-terminal-α-thioester and another unprotected peptide with a N-terminal Cys residue determines the final formation of a native peptide bond at the ligation

site. As shown in Fig. 9, at first the nucleophilic thiol moiety of the Cys residues attacks the Cα of the thioester determining the exit of the SR- leaving group (R = alkyl group). Then, the intermediate undergoes a spontaneous, intramolecular, rapid and irreversible rearrangement, via a favorable five member transition state, that forms an amide bond and restores the Cys residue.[38]

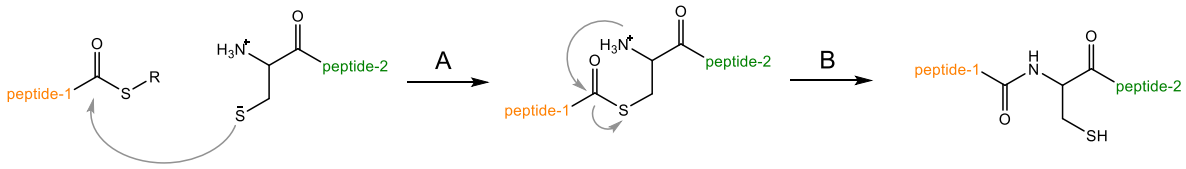


*Fig 9: Schematic representation of the Native chemoselective ligation. Where R is a generic alkyl group (**A**) Capture through a nucleophilic attack of the thiol (**B**) Spontaneous rearrangement that determines the formation of an amide bond.*

The success of this reaction its attributable to its inherit selectivity that derives from irreversible rearrangement. In fact, the presence of another Cys moiety in the peptide chain would not interfere with the ligation: the thiol-thioester exchange takes places either way, but when there is not a nearby terminal free amine, the reaction is a reversible equilibrium. Therefore, the overall equilibrium is driven to form the amide bond between the C-terminal-α-thioester and the N-terminal of Cys as described above.[34, 39] Moreover, the mild reaction conditions, of neutral pH in a aqueous buffer, in which the reaction takes place are ulterior advantages of this reaction. The same reaction can be performed with the selenium analogue of Cys (Sec). Indeed, the SeH group is more acidic and nucleophilic than its thiol counterpart, therefore NCL ligations are very fast and occur in high yield.[39] On the other hand, Sec is more easily oxidized and exists only in its dimeric form, thus a reduction reagent is required for NCL reaction to proceed efficiently.[40]

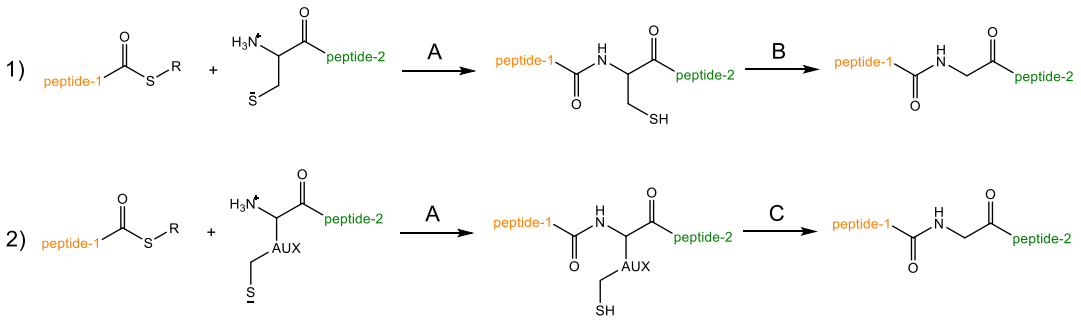


*Fig 10: Non-Cys Native chemoselective ligation. (**A**) Chemoselective ligation in the presence (2) or in the absence of the auxiliary (AUX) (1); (**B**) Desulfurization; (**C**) Removal of the auxiliary group*

This type of ligation was further improved when the post-desulfurization of the Cys moiety and the use of a removable thiol containing Nα auxiliaries where introduced (Fig.10 and 11). In fact, both cases determine the insertion of an Ala residue at the ligation site instead of Cys, that is less frequently present in natural proteins.[34, 39] Specifically Cys has a 1.8% abundance in comparison with the 8.9% of restudies of Ala.[40] At first the

desulfurization procedure involved Raney Ni or Pd/Al_2O_3 catalysts. To avoid side-reaction at some side-chain groups, a metal free desulfurization with the radical initiator VA-044 and TCEP was developed.[41]

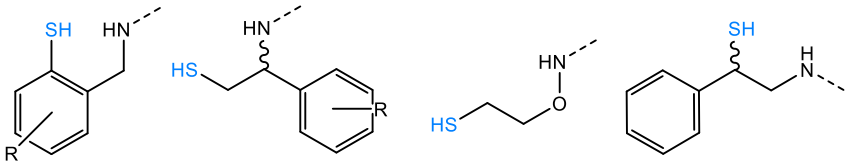


Fig 11: Some examples of thiol containing Nα auxiliaries (AUXs)[40]

Non-Native Chemical Ligation

The chemoselective ligation is not limited to NCL. In fact, a wide number of non-native ligations have been re-discovered in the last years. Some of the most relevant to date, are listed in the following table:

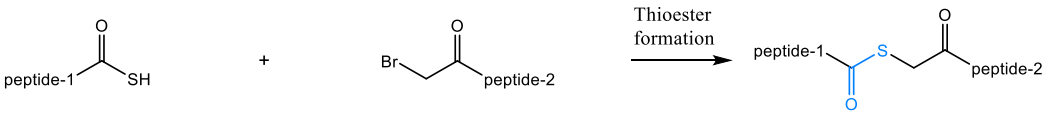
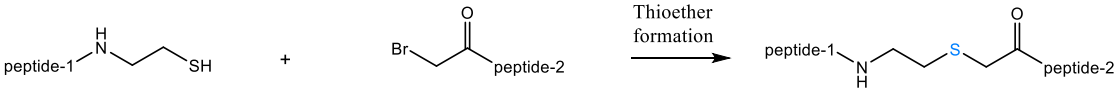
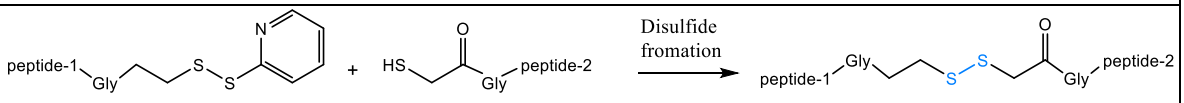
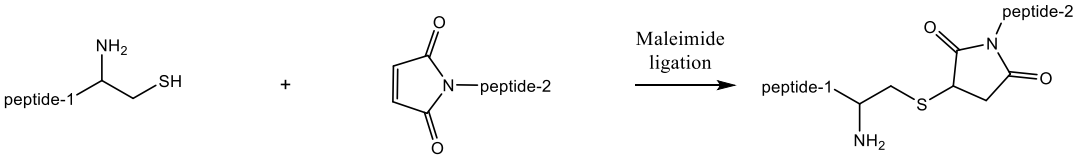
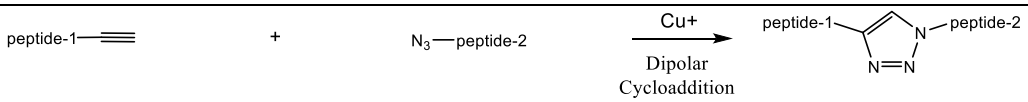
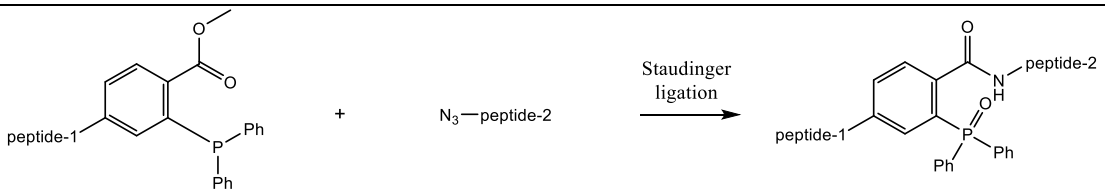
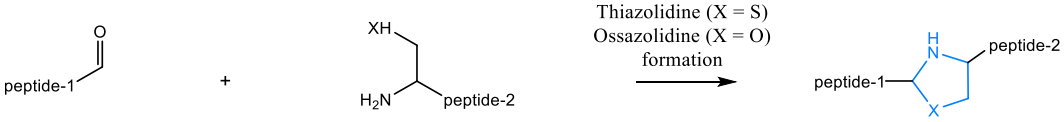
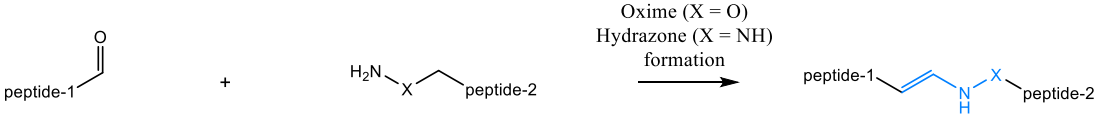
Ref.	Reaction
[35]	
[42]	
[43]	
[44]	
[45]	
[46]	
[47, 48]	
[49, 50]	

Table 1: some of the most relevant non-native chemoselective ligations.

Cysteine is one of the preferred attachment sites among canonical amino acids. In fact, the nucleophilicity of the thiol moiety makes it highly reactive especially at physiological pH. Consequently, coupling via the cysteine side chain is a highly attractive method.[44] Historically the first use of thiol moieties in the context of chemoselective ligation is in the synthesis of HIV-1 protease analogue. The thioester bond and the disulfide linkage were used to merge the two segments of a subunit and the two subunits together, respectively.[35, 43] The specific and efficient coupling of maleimides with S-alkyl thiols makes them most popular partner for this type of conjugation.[44] The drawback of using natural side-chains groups for the functionalization of proteins is that site-selectivity cannot be achieved when more than one is present within a protein.

Therefore, a useful approach would be the introduction of a unique functional "handle" not naturally present in the protein.[9] Examples of this strategy are dipolar cycloaddition and the Staudinger reaction. In the first, copper ions catalyze the reaction between an alkyne and an azide. To overcome the toxicity of copper to cells, a strain-induced cycloaddition reaction was developed; but this reaction was also characterized by slow rate and a lack of regioselectivity. The Staudinger ligation has been employed in many applications (ex. labelling of proteins and DNA or immobilization of proteins to surfaces), and is not only chemoselective, but also biocompatible with living systems.[36]

Chemical Ligation with Aldehydes

Another functional group not traditionally found within proteins is the aldehyde functionality. Oxime and hydrazone ligations are the most frequently used method for modifying protein aldehyde handles (Table 1).[9] On the other hand, the reaction of aldehydes with Cys and Thr, to form heterocyclic structures has been known for decades but, despite its inherit advantages, has not been explored properly (Table 1).[51]

Oxime and Hydrazone ligations

Oximes and hydrazones are known since the end of the 19th century.[52, 53] Currently they are extensively applied for bio-conjugations in systems that span from the decoration of nanoparticles to the synthesis of molecular switchers. The success of this ligations is attributable to the small size of the bond, which does not interfere much with the structure of the native biomolecule; the reversibility of the conjugation, that can be exploited for the controlled release of biomolecules; the low interference with biological processes and metabolites; and the fact that the only byproduct is water.[54] The oxime and hydrazone ligations need an alkoxyamine and a hydrazine (α-nucleophile) to react with a carbonyl moiety, respectively.

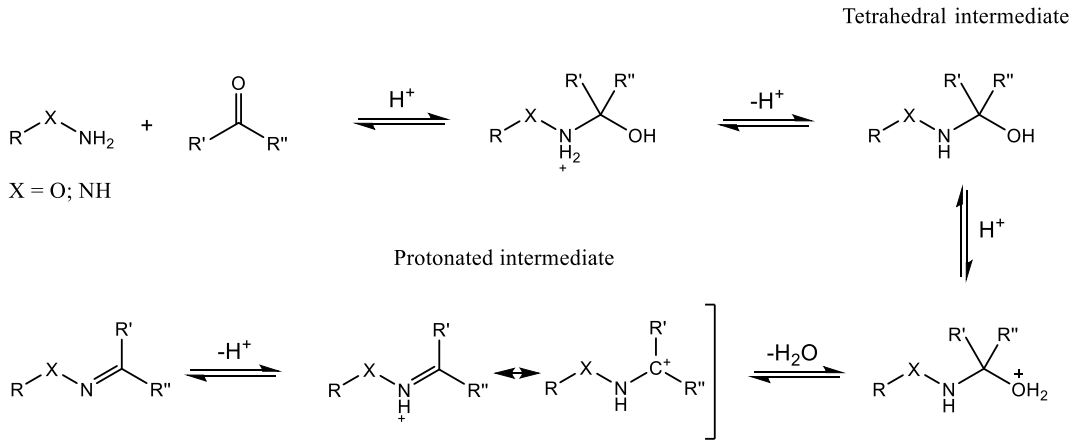


Fig 12: Mechanism of oxime and hydrazone formation. Starting from an alkoxyamine/hydrazine and an aldehyde/ketone. Where R is the linking group and R' and R'' are generic alkyl groups. Oxime: X = O. Hydrazone: X = NH.[54]

As shown in Fig. 12, the conjugation if fully reversible. The reaction starts with a proton-catalyzed attack of the α-nucleophile to the carbonyl atom. The tetrahedral intermediate obtained upon proton transfer undergoes dehydration and determines the formation of a protonate intermediate and water. The final deprotonation of double bonded resonance form of the dehydrated product determines the formation of the final product. Due to the catalytic role of the proton, the reaction is typically performed at acidic pH (Fig.13)[54]:

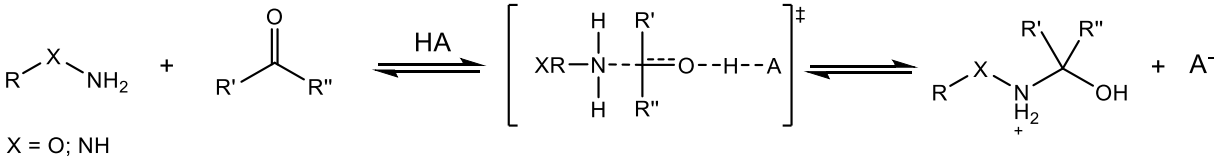


Fig 13: Proton-catalyzed attack of the α-nucleophile to the carbonyl atom. The transition state proposed by Kolmel et al. is also illustrated.[54]

At pH from 3 to 7 the dehydration of the tetrahedral intermediate is the rate determining step. But at a pH lower than 3 the nitrogen atoms of the α-nucleophiles tend to be protonated. Thus, also the nucleophilicity is lower and the first nucleophilic attack becomes the rate determining step. Taken all this into account, a pH of 4.5 is typically ideal for the formation of oximes and hydrazones. The back reaction is initiated by a protonation of the imine nitrogen. Also, the higher the electronegativity of X (with X = O for the oxime and X = NH for the hydrazone) is, the lower the basicity of the imine nitrogen. Thus, thanks to the higher electronegativity of the oxygen atom, the oxime generally is a more stable linkage than the hydrazone. The stability depends also on the carbonyl counterpart of the reaction: the higher the electrophilicity the more stable is the ligation, that is why aromatic aldehydes are frequently used for this type of reaction.[54]

Thiazolidine and Oxazolidine ligations

In 1994 Liu *et al.* first described the use of the thiazolidine and oxazolidine for the ligation of two peptide segments.[47, 48] The goal behind their work was to overcome the entropy barrier of the linkage of two large molecules through a highly specific reaction and without side-chain protection.[47]

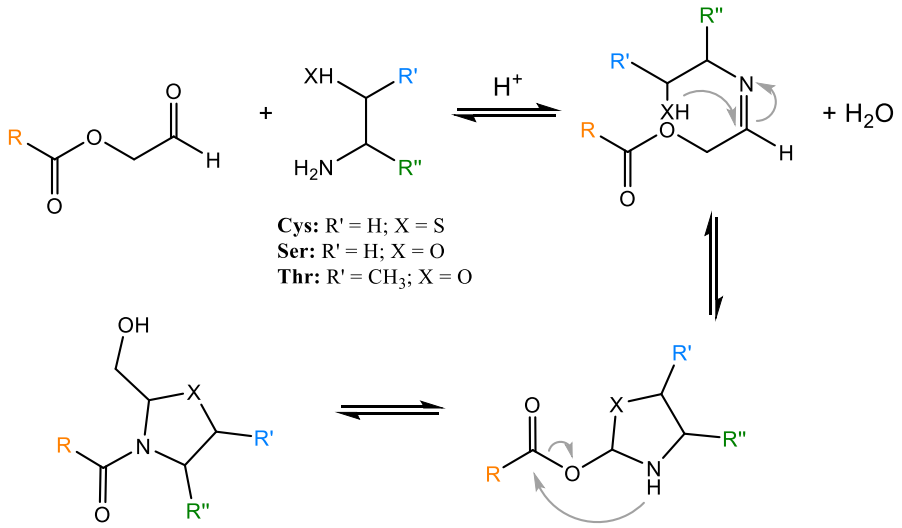


Fig 14: Mechanism of thiazolidine and oxazolidine formation. The first step is the imine capture: the nucleophilic Nα-terminal amine attacks the Cα-terminal with the consequent elimination of water and the formation of an imine. The second step is the thiazolidine (X = S) or oxazolidine (X = O) ring formation. At the end, a O- to N-acyl transfer determines the formation of the final proline like product.

The selected reaction involves an alkyl aldehyde and a weak base, in particular a β-amino thiol or hydroxyl moiety. As shown in Fig. 14, the highly specificity lies in the formation of a stable five membered ring. In fact, only the moieties of Cys, Thr and Ser are able to form the ring, whereas the concurrent reaction with an amine Schiff base would be reversible and unstable. Also, since the ideal pH for this linkage is 4.5, the reaction of aldehydes with other nucleophiles are prevented by protonation. Specifically, the Nα-terminal amine attacks the Cα-terminal to form an imine bond with the elimination of a water molecule. Then, the side chain nucleophile attacks the electrophilic carbon forming a five membered ring. In this case, the aldehyde was introduced at the C-terminal of a peptide sequence, therefore the favorable proximity of the ester-carbonyl and the Nα-amine determines the subsequential intramolecular O- to N-acyl transfer and the formation of an amide bond. The obtained final product has a proline mimetic structure.[47] The back reaction is triggered by the presence of electrophiles, that is why this type of ligations are also used as temporary protection of Cys in peptide synthesis.[47, 48] In particular, Cys is the most reactive towards this ring formation. In fact, it is able to undergo a complete and fast conversion in 100% aqueous and water-miscible organic solutions in a pH range of 5-8. In aqueous conditions the oxazolidine formation is 100-fold slower in the case of Thr. But, in polar aprotic solvents the Thr manages to complete the conversion rapidly. Ser reacts very slowly and incompletely

both in aqueous and organic solvents. This type of reaction was also tested with His, Trp or Asn. The first two react similarly to Ser, whereas the last does not substantially react in either solvent. In these cases the ring formation was not reversible.[48]

The thiazolidine, oxime and hydrazone ligations were compared in a dendrimer synthesis study. Since all three reactions start with an elimination of water molecules after the nucleophilic addition, they demonstrated that their reaction rate is improved in polar aprotic solvent mixtures. Also, all three reactions are pH dependent and in a general range of 4 to 6 the reaction rate increases with the increase of the pH. Of particular interest, in the case of Cys, is the formation of the disulfide product that diminishes the reaction rate at higher pH. Furthermore, also a higher temperature (tested up to 37°C) increases the reaction rates. Especially in the hydrazone case. The thiazolidine ligation product is stable in a pH range of 3-9, whereas oxime is not stable in basic environments and hydrazone is only stable in a 5-7 pH. Overall, thiazolidine was demonstrated to be the more stable linkage with the fastest reaction rate.[55] Thus, the rapid reaction rate, the stable product formed also in aqueous solvents and the catalyst free ligation makes thiazolidine a highly attractive type of click reaction.

2.3 PEPTIDES

Peptides are organic molecules made of a short number of amino acids joined by amide bonds. The amino acid composition and sequence determines the peptide activity. According to their mode of action they can be generally classified into: antioxidants, immunomodulatory, cytomodulatory, opioid, mineral-binding, antihypertensive, antithrombotic and antimicrobial.[56]

Antimicrobial Peptides

The first antimicrobial peptide (AMP, also known as host defense peptides of HDPs) to be discovered was *gramicidin* in 1939. This peptide was, indeed, able to protect mice from Pneumococcus infections.[57-59] It was then discovered that bacteria, fungi, plants, invertebrates, arthropods, fishes, amphibians, reptile, birds and mammals are all producers of AMPs as part of their innate immune response to viruses, fungi, bacteria, but also protozoa and even cancer cells. [60] Due to their potential multifunctional activity AMPs are often called "natural antibiotics". Also, their broad-spectrum mechanism of action makes them at low risk of resistance development and thus promising for pharmacological applications. Even in a synergetic administration with classical antibiotics.[8]

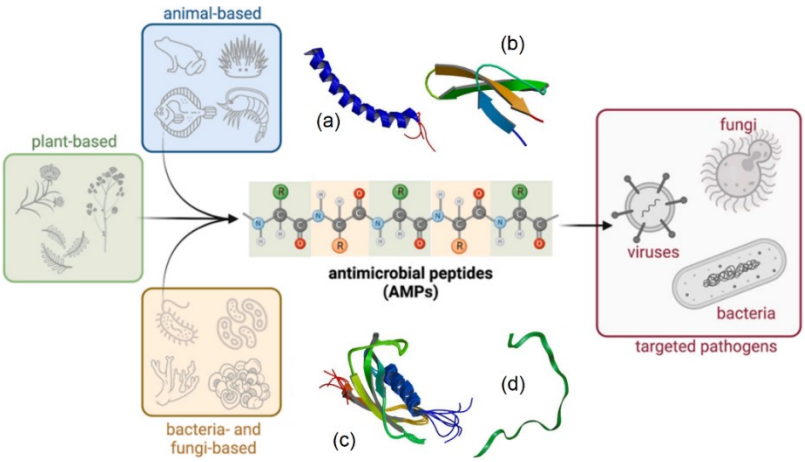


Fig 15: Schematic representation of the origins, the targets and the most common structures of AMPs.(a) α-helix; (b) β-sheet;(c) mixed α-helix and β-sheet ; (d) linear. Image modified from references [8, 61].

Although there is evidence of the existence of some anionic AMPs (charged from -1 to -2), the most abundant and studied class of AMPs are cationic antimicrobic peptides. They are a quite heterogeneous class but they are still accumulated by some distinctive characteristics:[62, 63]

- Small size: from 10 to 25 amino acid residues and 1-5 KDa. The length of the chain can count up to 60 residues but usually, shorter AMPs are preferred in clinical treatments for the lower costs and cytotoxicity.

- Cationic (Arg and Lys) and hydrophobic residues (with aliphatic and aromatic side chains) as the main constituting types of amino acids.
- Tendency to adopt amphipathic structures
- A net positive charge that ranges from +2 to +9.

From this point on only cationic AMPs are taken in consideration when referring to AMPs (if not differently noted). The most used classification of AMPs is based on the secondary structure: α-helixes are the most common, followed by β-sheets but also mixed and linear structures are present (Fig.15). It was also demonstrated that in water, the majority of AMPs are in a disordered state but in membrane mimetic environments they undergo a conformational change and assume their secondary structure.[8]

Mechanism of action

Based on the mechanism of action AMPs are divided into membrane-targeting and non-membrane targeting AMPs. The first act damaging the structural integrity of the cell membrane, whereas the others exert their antimicrobial properties inhibiting essential cell components or cellular functions.[8]

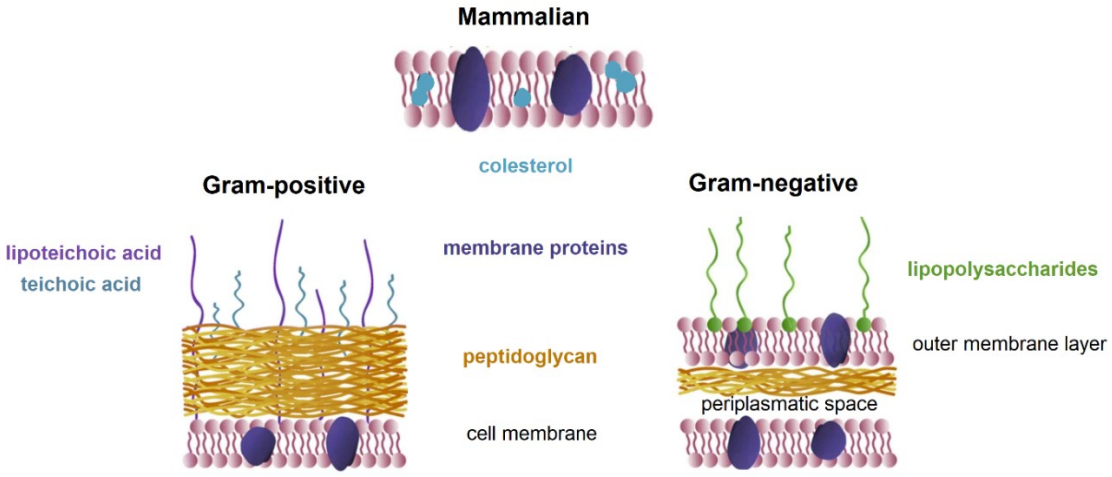


Fig 16: Schematic representation of mammalian plasma membrane and bacteria membranes.
Taken apart the functional and structural diversity of cell membranes, all of them are effectively planar, with a thickness between 50 Å and 100 Å, manly composed of amphiphilic lipids and proteins. In the plasma membrane of mammalians four major phospholipids predominate: zwitterionic phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and negatively charged phosphatidylserine (that are located in the inner leaflet of the membrane). They also contain a large amount of cholesterols and glycerolipids and some inositol phospholipids that are important in cell signaling. Gram positive bacteria have one cell membrane that is surrounded by a thick wall of peptidoglycan. Teichoic and lipoteichoic acids are embedded in the cell wall. Gram negative bacteria present a thin layer of peptidoglycan between the inner and outer membrane. Lipopolysaccharides are the major component of the outer leaflet. The main lipid components of bacteria are the negatively charged phatidylglycerol and cardiolipin.[64] Images modified from [65]

The electrostatic attraction between the opposite charges of AMPs and bacterial membrane is the first step for the antimicrobic action of the membrane-targeting AMPs. This is strengthened also by hydrophobic interactions.[8] Thus, the selectivity of these AMPs lays in the differences between mammalian plasma membranes and bacteria membranes. For mammalians the negative charges are not exposed to the peptides to be perceived. In fact, the anionic lipids are located in the inner leaflet of their plasma membrane. Also, the presence of cholesterol in both leaflets increase the membrane thickness and density making more difficult for the peptides to penetrate. On the other hand, bacteria do not have sterols in their membranes and at least 15% of their lipid components are anionic. Thus, bacterial membranes tend to be highly negatively charged and therefore attractive to AMPs[64] (Fig. 16).

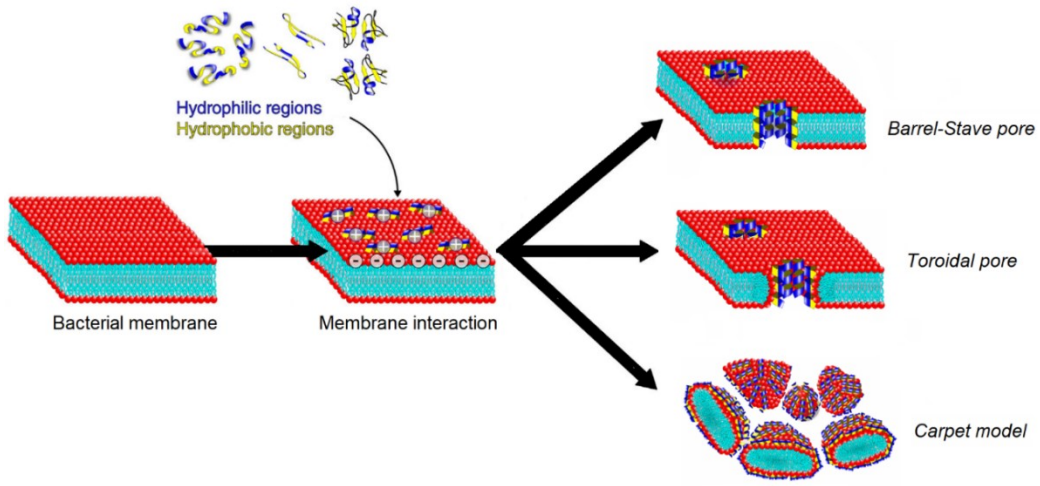


Fig 17: Mechanisms of action for membrane-targeting AMPs[60] They all are accumulated by the initial attraction to the bacterial membrane due to electrostatic and hydrophobic interactions.

Upon accumulation of AMPs, the membrane reaches critical aggregation concentrations and AMPs begin to affect the membrane integrity.[8] The three principal identified models of action are (Fig. 17):

- *Barrel-stave pore model:* first the peptides attach to the membrane surface parallelly. Then the interaction between the hydrophobic regions of the peptides with the inner hydrophobic core of the membrane causes their insertion and the formation a transmembrane pore. In this model the hydrophilic regions of AMPs face the inner part of the channel.[66]
- *Toroidal pore model:* the pore formation in this case is triggered by a peptide-lipid interaction and not by the self-assembling interaction of the peptides. This causes a local curvature of the bilayer and the consequent formation of a toroidal pore.[66]
- *Carpet model*: the peptides accumulate and spread all over the membrane surface in a parallel arrangement. The reaching of a critical concentration causes the reorientation of the peptides towards the inside of the membrane (similarly to the toroidal pore model) and the degeneration of the membrane into smaller micelles with a hydrophobic core.[67]

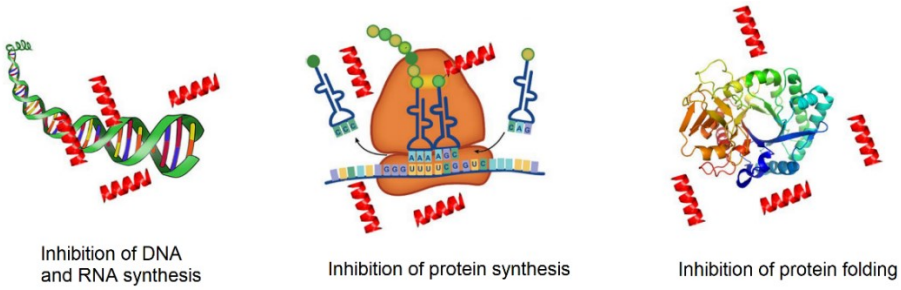


Fig 18: Schematic illustration of some mechanisms of action for non-membrane targeting AMPs[8]

However, many AMPs are proven to act with different mechanisms that do not involve the bacterial membrane as their primary target. The so-called non-membrane targeting AMPs first translocate into the cytoplasm without damaging the membrane. Then, they can inhibit various critical cellular processes (Fig. 18).[8] For example, the proline-rich AMPs mostly act inhibiting the bacterial protein synthesis.[68] Some other AMPs interfere with the synthesis of nucleic acids[69] or the correct folding of essential proteins[70]. It was also demonstrated that a group of AMPs act as antibiofilm agents in the case of biofilm-related infections.[71]

PMAP-36 and analogues

One interesting example of AMPs are the porcine myeloid antibacterial peptides (PMAPs). Up to date, three of this cathelicidins have been discovered: with 23, 36 and 37 amino acidic residues (PMAP-23, -36 and -37), respectively. PMAP-37 and -37 adopt an amphipathic α-helix whereas PMAP-23 assumes an antiparallel β-sheet connected by a loop at center.[72] All three exert an antibacterial activity against both gram-positive and gram-negative bacteria, but PMAP-36 also exert an antifungal activity against *Candida*.[72] Although PMAP-37 is the more potent, PMAP-36 appears to be the more suitable candidate for further application. In fact, besides being the phylogenetically closest to the human cathelicidin LL-37[73], PMAP-36 does not show hemolytic activity to human erythrocytes up until 100 µM, whereas PMAP-37 does already at 10-50 µM [72].

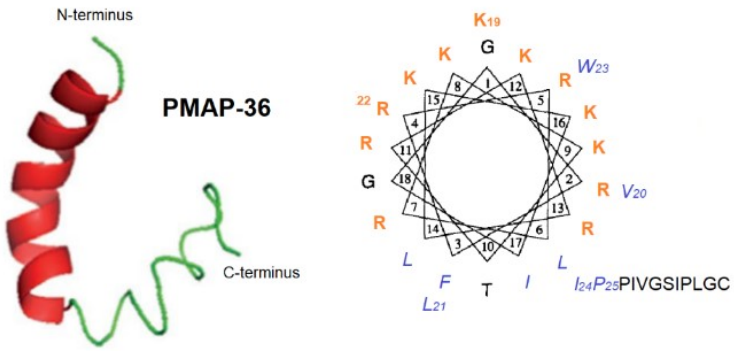


*Fig 19: **On the left:** secondary structure of PMAP-36.[73] **On the right:** helical wheel projection of PMAP-36 (1-20). Charged residues indicated in orange and bold, strong hydrophobic residues indicated in blue and italics. The projection concerns residues until Ile_{24}, all further residues are shown as a linear tail. [74] [75]*

PMAP-36 is the most cationic of the cathelicidins discovered so far.[75] As shown in Fig. 19, most of its cationic residues are located in the first 20 amino acids and the chemical synthesis of PMAP-36(1-20), permitted to demonstrate this part of the peptide to be an amphipathic α-helix. Also, PMAP-36(1-20) was proven to maintain in vitro antibacterial activity against several Gram-negative and Gram-positive bacteria.[74, 75]

The C-terminal end of PMAP-36 is rich in prolines, contains most of the hydrophobic amino acids and seems more loosely structured.[73] Furthermore, the thiol moiety of Cys^{35} allows the formation of dimers through a intramolecular disulfide bridge. The properties of the dimer were studied thanks to a shorter analogue: PMAP-$36(1\text{-}35)_2$. In the study it was demonstrated that dimerization favors helix formation, but it does not greatly affect the antibacterial potency in vitro or the cytotoxicity. However, the dimer has a greater capacity for long-term membrane damage, which determines a more efficient killing.[75]

Peptide	Sequence	Length	Charge
PMAP-36	H-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG-NH_2	36	+14
PMAP-36(1-20)	H-GRFRRLRKKTRKRLKKIGKV- NH_2	20	+13
PMAP-$36(1\text{-}35)_2$	(H-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGC- $NH_2)_2$	70	+28
PMAP-36(7-24)	H-RKKTRKRLKKIGKVLKWI-NH_2	18	+11
PMAP-36(13-24)	H-RLKKIGKVLKWI- NH_2	12	+6

Table 2: Sequences of PMAP-36 and some of its analogues. The NH_2 at the C-terminal underlines the chemically synthetic origin of the studied peptides.

It should be noted that PMAP-36 was proven to be mildly cytotoxic to murine macrophage-like cells and porcine erythrocytes.[73] Thus, studies has been made to diminish the cytotoxicity and at the same time reduce the costs of production with the development of shorter analogues of PMAP-36. Scheenstra *et al.* concluded that the truncation of 11 residues at the N-terminal does not affect much the antibacterial activity but reduces the cytotoxicity. But the deletion of four more amino acids decreases the activity. This is rationalizable when the mechanism of action is taken in consideration. In fact, the disruption of the inner membrane of bacteria is thought to be the way that of PMAP-36 kills bacteria and fungi, probably by toroidal pore formation at low concentration and following the carpet model at higher concentrations.[76] Therefore, the effect of the four missing residues could be due either to the diminished charge (from +6 to +3) or to the reduced length of the peptide that would impede the analogue to fully span the membrane to form pores.[73]

Lyu *et al.* argued that a charge of +6 is the threshold for driving peptides to the bacteria membrane via electrostatic interactions. They also found that the higher the charge and lower the peptide sequence, the higher the antimicrobic activity and the lower the cytotoxicity, respectively.[77] This could be done thanks to the synthesis of two other interesting short PMAP-36 analogues. Specifically, both the peptides C-terminals were truncated at the Ile^{24}, PMAP-36 (7-24) started from the Arg^7 whereas PMAP-36(13-24) started at the Arg^{13} (Table 2).

Solid Phase Peptide Synthesis

Nowadays chemically synthetic peptides are produced with the solid phase peptide synthesis (SPPS). This technique was invented by Merrifield in 1963.[78] The principle underlying this innovative method is the covalent attachment of the first residue of the amino acidic chain to a solid support; the addition of the following residues is done stepwise and when the sequence is complete the peptide can be removed from the solid support. In this way, filtration, washing and purification procedures are much easier. In fact, the growing peptide remains attached to the insoluble support whereas the excess of reagents, soluble byproducts and the solvents are filtered away. In contrast with the previous in solution methods, this technique allows a faster synthesis of peptides with higher molecular weights without concerns about the solubility of the products or transfer of material. Furthermore, the potentiality of being automatized makes it even more interesting.[78, 79]

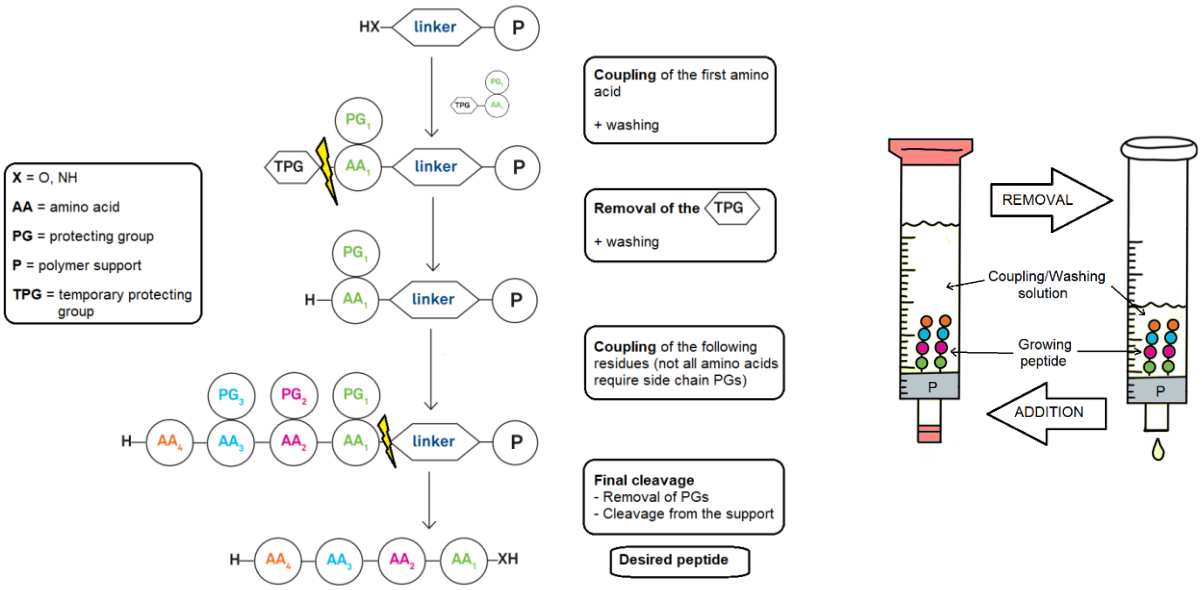


Fig.20: ***On the left:*** *Schematic representation of the SPPS principle. Image modified from [80]* ***On the right:*** *Schematic representation of the removal and addition of reagents excess/solvents in the SPPS. After the reaction/ washing takes place, the solutions are removed and only the growing peptide remains attached to the resin. Then the cycle can continue with a new refill.*

The SPPS usually follows the C-terminal → N-terminal direction for the synthesis and requires a series of repetitive steps to proceed (Fig. 20). First, the first amino acid is anchored to the polymer support. To do so, the resin have a free hydroxyl or amine residue that can react with the Cα-terminal of the amino acid. In fact, both the reactive side chains and the N-terminal α-amine are masked with permanent (PG) and temporary protecting groups (TPG), respectively. Usually, the peptide is added in an excess and in the presence of activating agents. to drive the reaction to completion. After the reaction takes place, the solution with the activated reagents is filtered away and the resin is washed several times (Fig. 20). Then, the TPG is removed in mild conditions and the following amino acid is attached. After the final residue is attached, the peptide is released from the resin and the sidechain protecting groups (PG) are concomitantly removed.[79]

However, there are some drawbacks to the use of SPPS. In fact, byproducts arising from either incomplete reactions, side reactions, or impure reagents will accumulate on the resin during chain assembly and contaminate the final product. Furthermore, the standard analytical techniques employed to follow the rection are generally not applicable (ex. TLC or mass spectra). To detect the presence of residual amines on the solid phase usually a colorimetric test is performed (Kaiser test, see below). In fact, the cleavage of small amount of peptide to check the correct growing od the chain with a mass spectrum, require much more effort than the mass spectra of a solution. Moreover, the yield of each coupling strongly limits the overall yield and the synthesis of a peptide with more than 50 amino acidic residues is virtually impossible, as shown in table 3.[81]

Nr of coupling	**Yield of each coupling**	
1	99%	90%
	Overall Yield	
10	90	35
20	81	12
30	74	4
40	67	1
50	61	<1

Table 3: Effects on the overall yield with an increasing number of residues with a yield for coupling that is less than 100%[81]

Resins

Crosslinked polystyrene is the most commonly used support used in SPPS. But there exist also crosslinked polyamide-based resins and composite polystyrene-polyethylene glycol-based resins that are much more hydrophilic.[79] For SPPS, these polymers are usually crosslinked with 1% divinylbenzene. The resins are usually purchased as small spheres that swell when added to the solvent, allowing the polymer network to expand and be exploited for the reaction.[82] This degree of cross-linking is optimal, in fact an higher level would reduce the swelling and a lower level would determine a loss in the mechanical stability of the resin.[80] Anyhow, the peptide does not grow directly on the surface of the support but is covalently bound to a linker that also protects the C-terminal from possible reactions. The percentage of loading of the linker vary from 0.5 mmol/g to 0.8 mmol/g, and usually larger peptides require a lower loading capacity.[79] Depending on the type of linker, the cleavage conditions and the functional group of the released C-terminal change. For example, linkers that possess an amino function as the reactive moiety for the C-terminal, upon the final acid treatment split off the peptide as an amide.[80]

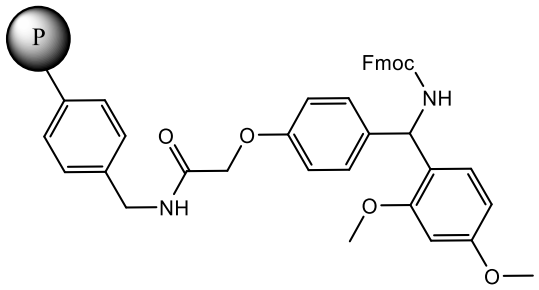


Fig 21: Fmoc Rink Amide AM resin. Cleavage conditions: 95% TFA

The resin used in this thesis is a Fmoc-Rink Amide AM. It is constituted of a 4-(2′,4′-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-aminomethyl linker bounded to crosslinked polystyrene.

Temporary protecting groups

Based on the protecting group of the α-amine, SPPS is divided in two strategies. The Boc/Bzl was the original strategy. Specifically, the Boc group protects the amine at the N-terminal and is removed by neat trifluoroacetic acid (TFA) at each step, whereas the other side chain protecting groups and the peptide-resin linkage are removed by treatment with anhydrous hydrofluoric acid (HF). Even though this method is highly efficient, the use HF determines two of the main disadvantages. The first is the need of special polytetrafluoroethylene-lined apparatus, that restrict this procedure only to specialists. The second is the risk of undermine the integrity of the peptide chains with fragile sequences.[79] The Fmoc/tBu strategy has become the method of choice in the routine synthesis. It is based upon an orthogonal protecting group strategy, whereas the former was based on a graduated acid lability. Specifically, Fmoc is labile to mildly basic environments while the other side chains and the bond at the C-terminal are acid-labile. This allows milder conditions for the final deprotection and cleavage.[79, 83]

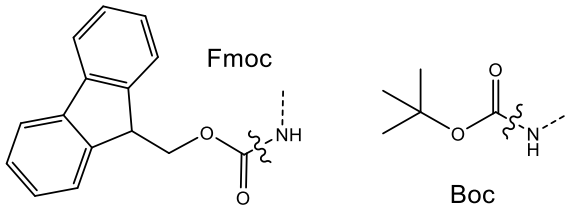


Fig 22: Chemical structures of Fmoc and Boc protecting groups.

The reaction of Fmoc with an amine determines the formation of a urethane bond which is not only easy to form and remove but it also prevents the racemization at the Cα. Specifically, a secondary amine, such as piperidine, breaks the urethane bond of Fmoc by a β-elimination (Fig. 23).

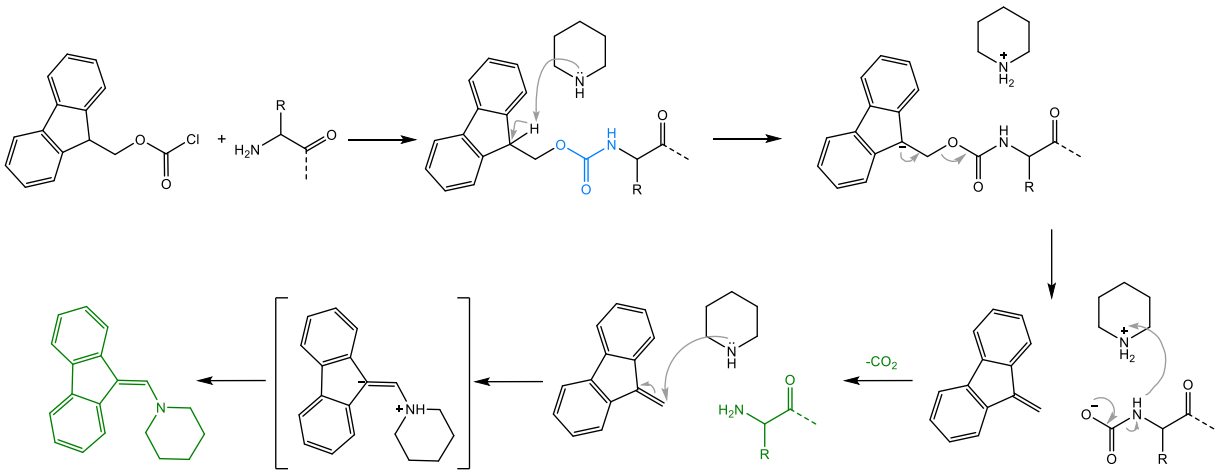


Fig 23: Removal of the Fmoc protecting group from a generic peptide by piperidine. In blue is shown the urethane bond. The overall reaction produces the free peptide, CO_2 and dibenzofulvene (shown in green).

Side chain protecting groups

Side chain protecting groups are needed to prevent side reaction with the reactive side chains of the amino acids. In the Fmoc/tBu approach side chains protecting groups have an orthogonal mechanism of release. Thus, they are stable at basic conditions and generally labile to TFA. They are removed at the end of the synthesis with the cleavage of the peptide from the resin. In this instance, the presence of additional molecules is needed. For example, triisopropylsilane (TIS) and water act as scavenger and are needed to neutralize the carbocations that are generated, whereas 3,6-dioxa-1,8-octanedithiol (DODT) prevent the formation of dimers when Cys is present. In the following table the side chains protecting groups used in this thesis are listed:

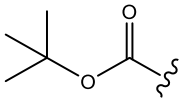
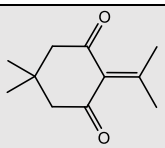
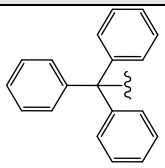
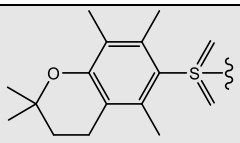
Amino acid	Name	Structure	Removal conditions[84]
Trp	Boc		95% TFA and scavengers
Lys			25-50% TFA-DCM
Lys	Dde		2% hydrazine-DMF
Cys	Trt		95% TFA and scavenger (TIS)
Arg	Pbf		90% TFA and scavengers (TIS and H_2O)

Table 4: Side chain protecting groups used in this thesis

Coupling reagents

The union of amine and carboxylic acid to produce an amide requires heating at 200°C to be spontaneous. But at this temperature the integrity of the peptide is compromised.[85] Thus, at room temperature it requires two steps. The first is the activation of the carboxyl group of one residue, usually converting the hydroxyl into a good leaving group. The second is the nucleophilic attack of the amino group to the activated carboxylic group.[86] Years of research have allowed the development of many coupling agents, the most-widely used are carbodiimides and phosphonium or aminium salts.[86]

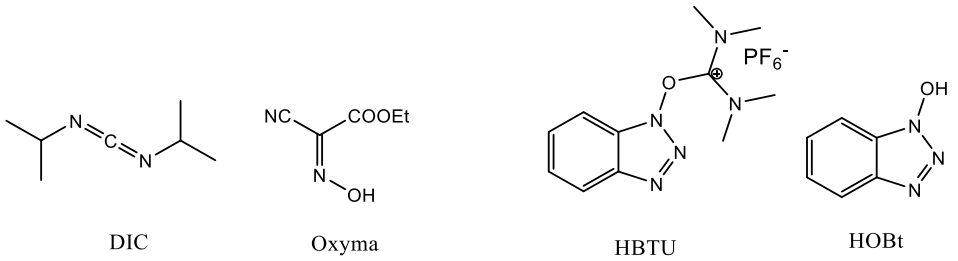


Fig 24: Chemical structures of the used coupling agents. ***DIC****, N,N'-Diisopropylcarbodiimide .****Oxyma****, ethyl 2cyano-2-(hydroxyimino) acetate.* ***HBTU****, N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate.* ***HOBt****, 1-hydroxy-1H-benzotriazole.*

In this thesis two systems were used to activate the coupling.

- **HBTU/HOBt system**. HBTU is an aminium salt and, especially in the presence of HOBt, was demonstrated to have low levels of epimerization. In this coupling, the activation takes place in the presence of a tertiary base, like the N,N-diisopropylethylamine (DIPEA) and causes the formation of an activated ester. The two coupling reagents are added before the DIPEA to avoid the formation of a Schiff base. This reaction is particularly efficient and is often used in routine synthesis. But it is important to underline that HOBt is potentially explosive.[85, 87]

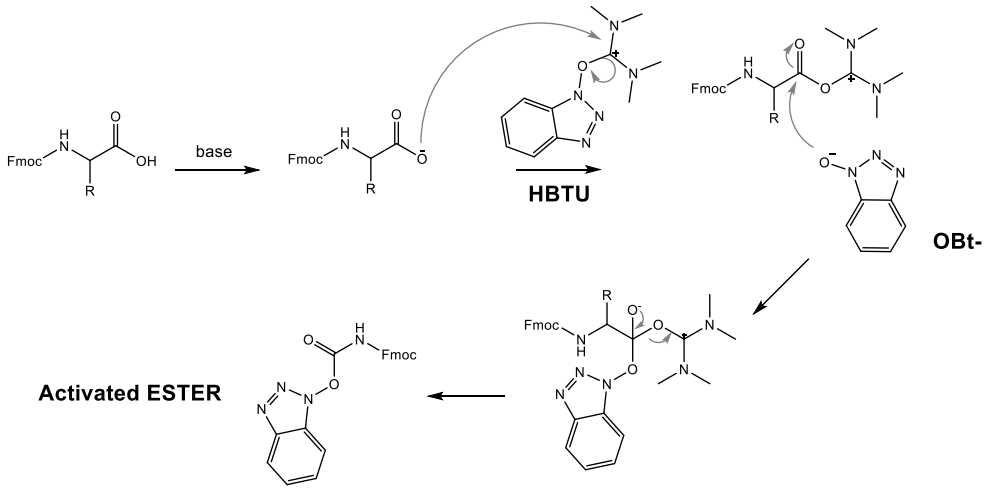


Fig 25: Activation of the carboxylic moiety of a Fmoc-protected amino acid by HBTU/HOBt system. The amino acid first reacts with HBTU to form the tetrametylurea , immediately after the activates ester is formed by OBt-. R is the protected side chain of a generic amino acid[85]

- **DIC/Oxyma system.** DIC is a classic carbodiimide agent that presents a level of amide formation comparable to HBTU,[85] whereas Oxyme, besides avoiding the above-mentioned explosive issues, was proven superior to HOBt in terms of suppression of racemization.[88] Also in this case, the activated carboxyl is an ester.

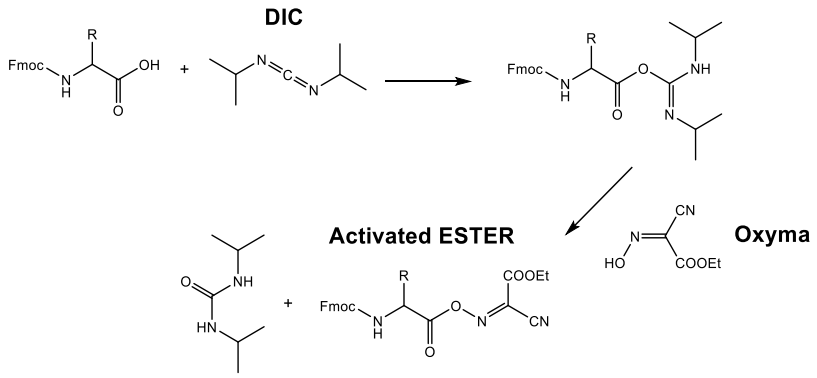


Fig 26: Activation of the carboxylic moiety of a Fmoc-protected amino acid by DIC/Oxyma system. R is the protected side chain of a generic amino aci [86]

Kaiser test

The Kaiser test[89], is used for the detection of free amines. The principle behind it is the fact that in specific conditions (a solution 1:1:1 of ninhydrin, potassium cyanide in pyridine and 80% phenol in ethanol) and upon heating, ninhydrin reacts with free amines to form a colored molecule named Ruhemann's purple[90] (Fig. 27).

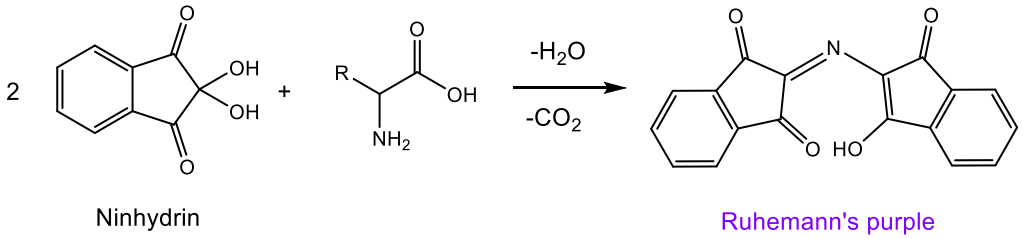


Fig. 27: Reaction between ninhydrin and an amino acid to form the Ruhemann's purple.

This test is regularly used in the SPPS to check if a coupling is successful. When there are not free amines the solution remains yellow. On the contrary, when there are amines, the solution becomes blue, and the test is considered positive. In this thesis the Kaiser test was used both to check the SPPS and to test the presence of peptides on the cotton surface.

3. RESULTS AND DISCUSSION

3.1 AIM OF THE THESIS

The aim of this thesis is the study of the conjugation between peptides and cotton for the development of antimicrobial textiles. The chosen method of conjugation was inspired by the concept of chemoselective ligation: unique and mutually reactive moieties are introduced both in the cotton weft and in the peptide chain. Considering the vast presence of hydroxyles groups on cotton, the aldehyde is the chosen reactive moiety; whereas Cys and Aox are the two chosen reactive groups for the peptides.

Thus, the thesis work can be rationalized into three main parts:

1) The study of the cotton oxidation in different conditions and on different cotton types.
2) The preparation of small "probe peptides" that serve as models to setup an efficient conjugation methodology for the covalent linkage between oxidized cotton and peptides.
3) The application of the conjugation protocol in the case of an antimicrobial peptide to produce an antibacterial textile prototype.

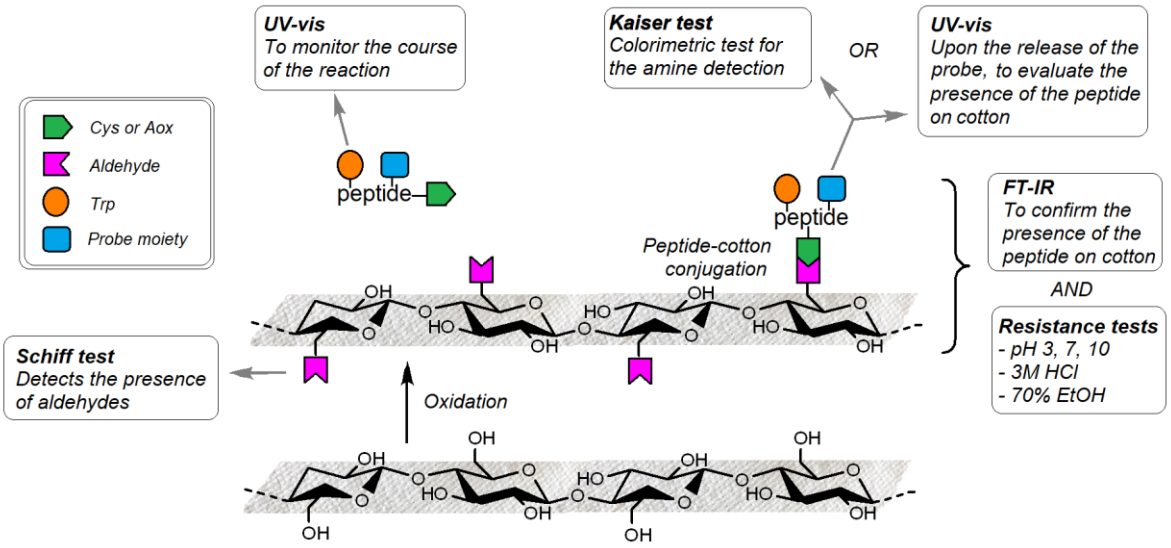


Fig.28: Schematic representation of the process used to produce and study thiazolidine and oxime conjugations in this thesis. All the used model peptides are equipped with the reactive group (Cys or Aox, shown in green), a UV active moiety (Trp, shown in orange) and probe groups (free amine or Fmoc group, shown in blue).

In more details (Fig. 28):

1) To introduce the aldehyde, cotton is oxidized by TEMPO/laccase/O_2 system. This method is chosen because it takes place in an aqueous solvent and preserves the integrity of the cellulose chain while avoiding the use of halogenated molecules.

2) This work studies the thiazolidine and oxime bond formation between oxidized cotton and peptides. Thus, in order to be able to perform the conjugation, Cys and Aox need to be introduced as the N-terminal amino acid of the target peptide chain. This is easily achievable through the SPPS. To investigate these peptide-cotton ligations, the following model peptides, that we address as "probe peptides", were prepared:
   - **C1**: H-Cys[1]-Gly[2]-Trp[3]-Lys[4]-NH_2
   - **A1**: H-Aox[1]-Gly[2]-Trp[3]-Lys[4]-NH_2
   - **A2**: H-Aox[1]-Gly[2]-Lys[3](Fmoc)-Trp[4]-Lys[5]-NH_2

   Considering that the effectiveness of the thiazolidine (Cys) conjugation of AMPs to cotton has already been proven[6, 7]; whereas the oxime bond formation (Aox) is widely used in bio-conjugations (see paragraph 2.2), but has never been exploited in this specific context. As concerning the peptide-cotton ligations, this thesis work focuses on:
   - Reactions that take place in water in the context of sustainable processes.
   - Setting the optimal conjugation conditions and comparing thiazolidine and oxime bond efficacy.
   - Quantifying the aldehyde content achievable on different types of cotton in order to maximize the cotton functionalization.
   - Testing the resistance of the two ligations, for the development of durable functionalized new textile materials.

3) The results obtained with the probe peptides are used for the conjugation of and active peptide to cotton. The selected antimicrobial peptide is a shorter analogue of PMAP-36: a cathelicidin known for its large spectrum antibacterial and antifungal activity. The peptides prepared for this aim are the following:
   - **C2**: H-Cys[1]-Lys[2]-Arg[3]-Leu[4]-Lys[5]-Lys[6]-Ile[7]-Gly[8]-Lys[9]-Val[10]-Leu[11]-Lys[12]-Trp[13]-Ile[14]-NH_2
   - **A3**: H-Aox[1]-Lys[2]-Arg[3]-Leu[4]-Lys[5]-Lys[6]-Ile[7]-Gly[8]-Lys[9]-Val[10]-Leu[11]-Lys[12]-Trp[13]-Ile[14]-NH_2

To confirm the structure of the probe peptides, NMR and HPLC-MS techniques are employed. The course of the reaction is followed through the UV-vis technique. The functionalized fabrics were analyzed by colorimetric tests both to confirm the presence of the peptide and evaluate roughly the completeness of the reaction. Some selected cottons with the probe peptide bound to them were subjected to FT-IR analysis as an ulterior confirmation of the presence of the peptide on cotton. Moreover, the cottons functionalized with **C2** were tested to determine their antimicrobial properties.

3.2 TYPES OF COTTON

In this thesis three types of cotton textile were used (Fig. 29):

- **A-cotton**: a gauze cotton provided by Santex Spa.
    - It is white and presents a wide weft. Thus, its threads are easily removed from the tissue for colorimetric analysis.
    - Before its use, a fast process of mercerization was performed on it to remove the paraffin layer that covers the surface of the tissue (see paragraph 5.3).[6]
    - This type of cotton was preferentially used in previous experiments[82], thus it was the first to be tested for the conjugation with the peptides.

- **B-cotton**: cotton textile provided by Piave Maitex Spa.
    - It is white and presents a thick weft. Thus, it is not possible to remove single threads and the tissue needs to be cut for colorimetric analysis.
    - It was industrially subjected to a $NaOH/H_2O_2$ treatment. Thus, the mercerization process was not needed.

- **C-cotton**: raw cotton provided by Santex Spa.
    - It is slightly beige and presents a thick weft of thick threads. As **B**, it is not possible to remove single threads and the tissue needs to be cut for colorimetric analysis.
    - Before its use, it was subjected to a slow process (about 2 weeks) of mercerization to remove the high amount of waxes present on the surface of the textile.[6]

A-cotton ***B-cotton*** ***C-cotton***

*Fig. 29: Types of cotton used in this thesis. **(A)** Gauze cotton from Santex Spa. **(B)** Cotton from Piave Maitex Spa. **(C)** Raw cotton from Santex Spa.*

3.3 OXIDATION OF COTTON

The introduction of the aldehyde sites on the cotton fabric is performed through the TEMPO/laccase/O_2 catalyzed oxidation of the C_6-hydroxyl group present in the glucose subunit of cellulose. This method was chosen because it simultaneously preserves the integrity of the cellulose chain and avoids the use of halogenated reagents (see paragraph 2.1).

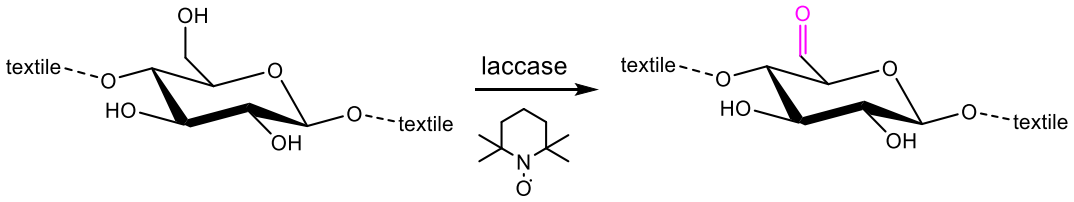
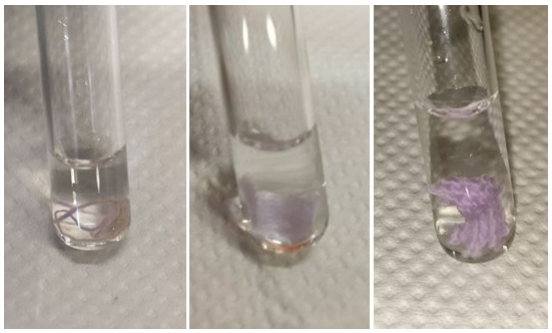


Fig. 30: Oxidation of cotton catalyzed by TEMPO/laccase/O_2 system (pH = 5).

All three types of cotton were subjected to this procedure and the reactions took place in an aqueous NaOAc/AcOH buffer at a pH 5. In order to maximize the aldehyde content, the reaction conditions (amount of laccase and TEMPO and reaction time) were those suggested by Aracri *et al*.[28] The cottons were oxidized in at total of 48 hours and the state of the reactions was checked performing a Schiff test on a small piece of cotton. As shown in Fig. 32, upon the Schiff reactant addition, oxidized cotton becomes pink whereas the not oxidized one remains white. Even though according to the literature the time required for the oxidation is of 20 hours, the reactions were performed for 48 hours. This was done because all cottons upon Schiff reagent addition after 20 hours of reaction resulted slightly pink.



A-cotton ***B-cotton*** ***C-cotton***

Fig. 31: Schiff tests after 20 hours with the spoiled Schiff reactant

Anyhow, the color of the test did not improve even after 48 hours. This can be explained by the fact that the employed Schiff reactant was later found out to be spoiled. Still, the change in the color of the cottons

suggested that the reaction effectively took place. A more quantitative evaluation of the amount of aldehyde sites is described at the end of paragraph 3.6.

Re-use of the oxidation mixture

Taking into account that this process is studied to produce a functionalized textile, the industrial point of view should also be considered. Thus, the possibility of re-use of the oxidation mixture was also tested. To do so, after the removal of the first piece of oxidized cotton, a second piece of cotton was added to the TEMPO/laccase solution, and the reaction was monitored as described above. The solution used for a specific type of cotton was re-used with the same type of cotton. All three types of cotton required four days to reach the same oxidation rate (a similar color of the Schiff test) of the first oxidation. The A-cotton solution was re-used for a second time and after four days the Schiff test resulted only slightly pink. Thus, the third oxidation was not performed for B- and C- cotton.

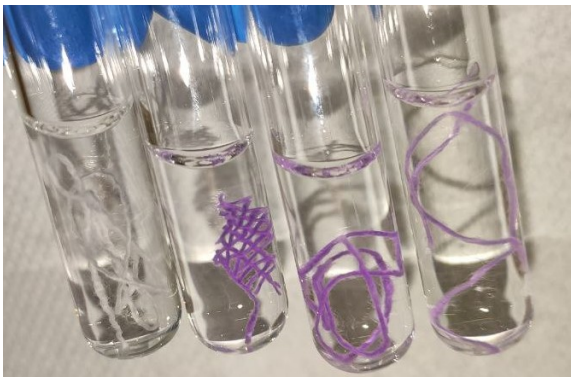


*Fig. 32: Schiff test with the fresh Schiff reagent on A-cotton. From the left: A-cotton, 1st oxidation of **A**, 2nd oxidation of **A**, 3rd oxidation of **A**.*

As shown in Fig. 32, the use of a fresh Schiff reagent later permitted to better appreciate the success of the oxidation in each re-use step. Upon multiple oxidations, there seems to be a slight decrease in the oxidation grade, that is better quantified in the following sections (see paragraph 3.6). This could be due to the proven degradation of the laccase enzyme by the oxoammonium ions; this degradation is however minimized when the laccase/TEMPO solution is in the presence of alcoholic moieties.[31] Overall , all the cottons were successfully oxidized by the TEMPO/laccase/O_2 system. Using a colorimetric method, there seems not to be a difference in the amount of oxidized sites for different types of cotton; but there is a difference when the oxidizing solution is re-used.

3.4 CHOICE OF PEPTIDES

Two types of peptides were used in this thesis: a series called “probe peptides” and an antibacterial peptide. The former were used to test the conditions for the conjugation with cotton, quantify the amount of peptide linked to cotton and evaluate the stability of the ligation in different media. Thus, the reaction conditions set with the probe peptides were checked with the antimicrobial ones and the antibacterial properties of the so obtained material were tested.

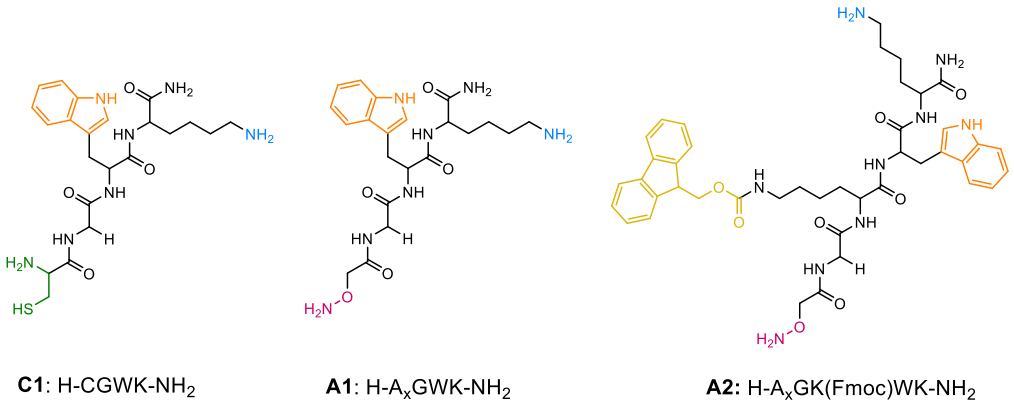


Fig. 33: Chemical structures of the probe peptides. Cys and Aox shown in green and magenta, respectively. Trp and NH_2 of Lys shown in orange and blue and Fmoc in yellow, respectively.

Probe peptides

Three peptides were designed to function as probes (Fig. 33). They all have an amino acidic chain of no more than five residues and present the following shared characteristics:

- **Short amino acidic chain**. The shortness of the peptides minimizes the steric hindrance at the ligation site when the peptide is bound to cotton. In this way, hopefully all the aldehydes on cotton should react with the peptides. Thus, the moles of bound peptide should correspond to the moles of aldehydes on cotton and can be quantified with a number ad not only in through colorimetric analysis. Furthermore, the shortness of the chain makes the peptide easy to synthetize and handle.

- A **reactive group selective for aldehydes** at the N-terminal that is needed for the chemoselective ligation with the oxidized cotton. In particular, **C1** ends with a Cys residue (green in Fig. 33), whereas **A1** and **A2** end with a Aox residue (magenta in Fig. 33). Thus, when reacting with the oxidized cotton, they form a thiazolidine and an oxime bond, respectively. The β-amino thiol and the alkoxyamine were chosen due to the ease with whom they can be attached to an amino acidic chain. In fact, one is the side chain of the

proteogenic amino acid Cys and the other the N-terminus of the non-natural amino acid Aox, both easy to purchase.

- The presence of **Trp** (orange in Fig. 33). A UV-vis reactive group is necessary to monitor the peptide-cotton conjugation reaction. Of the three natural peptides with an aromatic group (Trp, Phe and Tyr), Trp is the one with the highest extinction coefficient ($\varepsilon_{Trp\text{-}280nm\ in\ water}$ = 5630 M^{-1} cm^{-1})[91] and thus the chosen one. Specifically, the decrease in the absorbance at 280 nm of the peptide solution permits the quantification of the peptide that is no more in solution and thus bound to the cotton.

- The presence of **Lys** allows to confirm the presence of the peptide on the surface of the functionalized cotton. In fact, a Kaiser test performed on oxidized cotton results negative. But when an amine moiety (blue in Fig. 33) is bound to it, the Kaiser test turns blue.

- **Gly** as spacer between the chemoselective reactive group and the rest of the peptide.

The chemical structure of the probe peptides is shown in Fig. 33. **C1** and **A1** have the same last three amino acids (Gly, Trp and Lys) and differ only for the N-terminal peptide, which are a Cys and a Aox, respectively. On the other hand, **A2** was designed similarly to **A1** but with an additional Lys between Gly and Trp. In this way a Fmoc group (yellow in Fig. 33) could be linked to the additional Lys.
This was done to perform a double check on the amount of peptide bound to cotton:

- The first during the conjugation reaction thanks to the decrease of the absorbance of Trp (see paragraph 3.6)
- The second thanks to the absorbance of Fmoc ($\varepsilon_{Fmoc\text{-}289.8nm}$ = 6089 $M^{-1}cm^{-1}$)[92]. In fact, after the conjugation of **A2** to cotton, the Fmoc group can be removed from the peptide with a solution of piperidine. The concentration of dibenzofulvene in solution (formed upon Fmoc removal) allows the quantification of the peptide that is effectively bound to cotton.

Antimicrobial peptides

The antimicrobial peptide chosen for the conjugation with cotton is a PMAP-36(12-24), a shorter analogue of PMAP-36. The chosen segment of the goes from Lys^{12} to Ile^{24} of PMAP-36. Thus, the first 11 and the last 12 amino acids of natural PMAP-36 are not included in the sequence. At the N-terminal a Cys (**C2** or Cys-PMAP-36(12-24)) or a Aox (**A3** or Aox-PMAP-36(12-24)) is added as the chemoselective group that permits the ligation with the oxidized cotton (Fig. 34).

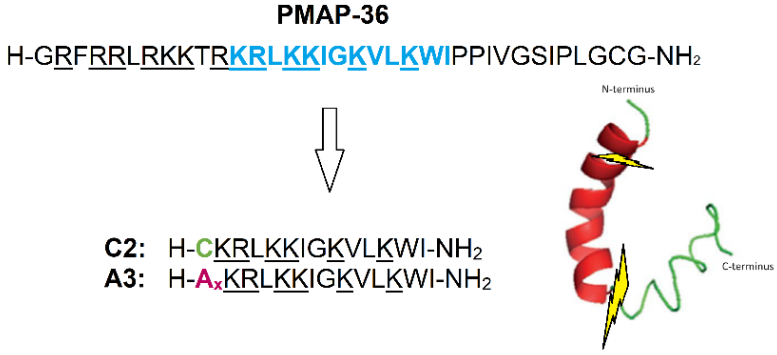


*Fig. 34: PMAP-36 and its PMAP(12-24) analogues. **C1** or Cys-PMAP-36(12-24) and **A2** or Aox-PMAP-36(12-24). Underlined are the charged peptides.*

This sequence is similar to the PMAP-36(13-24) used by Lyu *et al.* This amino acid sequence was proven to retain an antimicrobial activity, although to a lesser extent than the PMAP-36(7-24) portion, and also a low cytotoxicity.[77] In fact, it the removal of the C-terminal amino acids determines a lower cytotoxicity than that of the original PMAP-36. Furthermore, it was also proven (see paragraph 2.3) that the removal of the first 12 amino acids does not influence the antimicrobial activity of PMAP-36. Thus, the removal of the first 11 amino acids makes the synthesis more affordable in terms of costs without losses in activity. **C2** has one Lys more than PMAP-36(13-24). In this way the charge of the peptide is one unit more and probably this helps to improve the antimicrobial activity.

Peptide	Sequence	Length	Charge
C1	H-Cys^1-Gly^2-Trp^3-Lys^4-NH_2	4	+2
C2	H-Cys^1-Lys^2-Arg^3-Leu^4-Lys^5-Lys^6-Ile^7-Gly^8-Lys^9-Val^{10}-Leu^{11}-Lys^{12}-Trp^{13}-Ile^{14}-NH_2	14	+7
A1	H-Aox^1-Gly^2-Trp^3-Lys^4-NH_2	4	+2
A2	H-Aox^1-Gly^2-Lys^3(Fmoc)-Trp^4-Lys^5-NH_2	5	+2
A3	H-Aox^1-Lys^2-Arg^3-Leu^4-Lys^5-Lys^6-Ile^7-Gly^8-Lys^9-Val^{10}-Leu^{11}-Lys^{12}-Trp^{13}-Ile^{14}-NH_2	14	+7

Table 5: Summary table of the peptides synthetized in this thesis.

3.5 SYNTHESIS AND CHARACTERIZATION OF THE PEPTIDES

All the peptides described in the previous section were synthetized by SPPS using the Fmoc/Boc strategy. To determine the formation of an amide group at the C-terminal of the peptide chain, " Fmoc Rink Amide AM" resin was used as the solid support. This resin is purchased with the reactive amine protected with Fmoc, thus at the beginning of each synthesis a piperidine treatment is necessary to remove it. All the peptides were released from the resin with an acidic treatment with TFA. In this way also the side chain protective groups can be removed simultaneously. To purify the so obtained peptide solutions, the peptides were precipitated in cold Et_2O. When necessary, also a medium pressure flash chromatography was performed to further purify the peptides.

Peptide C1

Boc was used to protect the side chains of Lys and Trp, whereas Trt was used to protect the thiol moiety of Cys. The removal of the peptide from the resin was performed with a TFA solution in the presence of TIS, H_2O and DODT. The first two acted as scavenger and neutralized the carbocations that are generated in the release reaction (see paragraph 3.4). DODT prevented the formation of disulfide bridges between two Cys that would determine the formation of dimers.

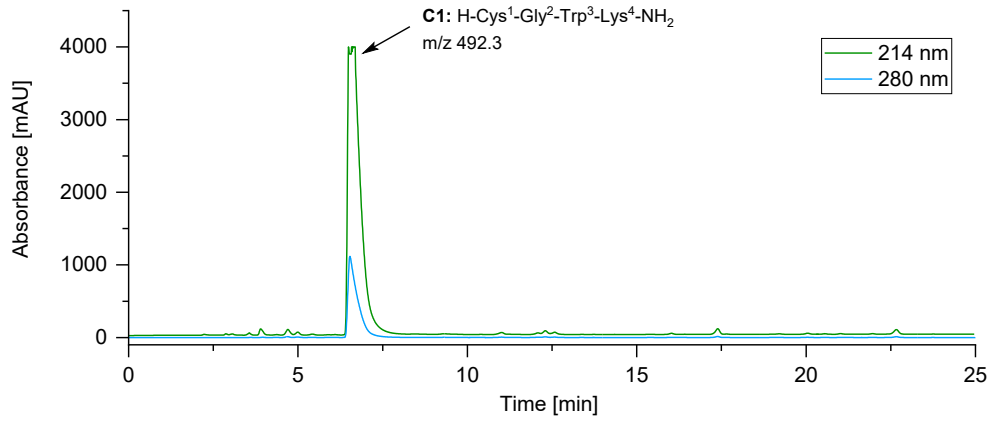


*Fig. 35: HPLC spectra of **C1.** Solvent H_2O + 0.1% TFA. Gradient of 0-30% of B in 30 min.*

The product was obtained with a high yield (see paragraph 5.4). After the reaction took place, an HPLC and a MS were performed to verify the purity of the solid obtained. To do so, the solid was dissolved in an acidic media to prevent the formation of the dimer. As shown in Fig. 35, only the target peptide was formed and there was no need of further purification.

Peptide A1

In this synthesis Boc was used as the side chain protecting group of for Lys and Trp, but also for the N-terminal of Aox. In this way, the N-terminal protecting group was removed together with the side chains protecting groups during the TFA treatment and one less piperidine treatment was performed. The release of the peptide from the resin was accomplished only in the presence of TIS and H_2O as scavengers, since there is no risk of forming disulfide bridges.

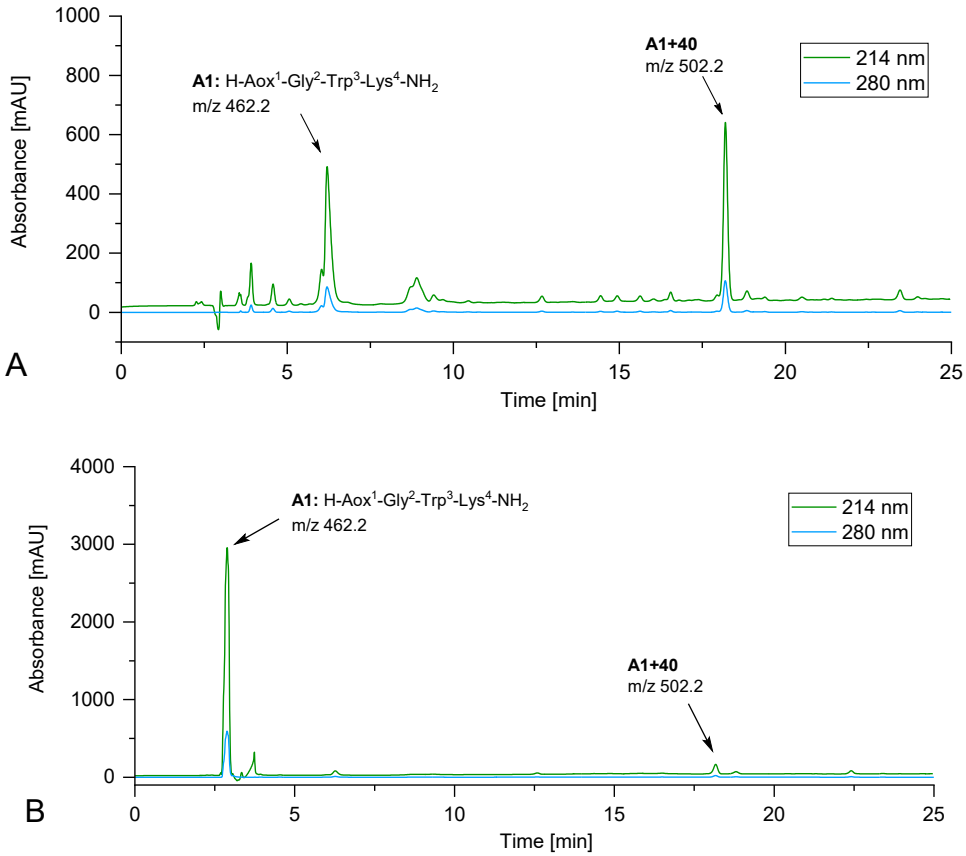


*Fig. 36: HPLC spectra of **A1.** **(A)**Solvent H_2O. Gradient of 0-30% of B in 30 min. **(B)**Solvent MeOH. Gradient of 0-30% of B in 30 min.*

After the reaction took place, an HPLC and a MS were performed to verify the purity of the solid obtained. As shown in Fig. 36, when the peptide was dissolved in water, a less polar byproduct with a m/z of 502.2 $[M-H]^+$ was formed. This byproduct was virtually absent when the same solid is dissolved in MeOH and, thus, there was not the need of further purification. This suggests that probably **A1+40** is not a byproduct of the synthesis, but it is formed when the peptide is in an aqueous solution.

Peptide A2

Similarly to **A1**, the used side chains protecting groups were Boc for Lys^5,Trp, and the N-terminal of Aox. The side chain of Lys^3 was protected with Dde, that is removable with hydrazine. In this way, after the synthesis of the complete amino acidic chain but before the release of the peptide, the Dde was removed without interfering with the other side chain protecting group. Then, the Fmoc group was linked to the amine of Lys^3, which was the only free one. At the end, also the N-terminal and the remaining side chains protecting groups were removed with the TFA treatment concomitantly with the release of the peptide from the resin. As in the case of **A1**, this was performed in the presence of TIS and H_2O as scavengers.

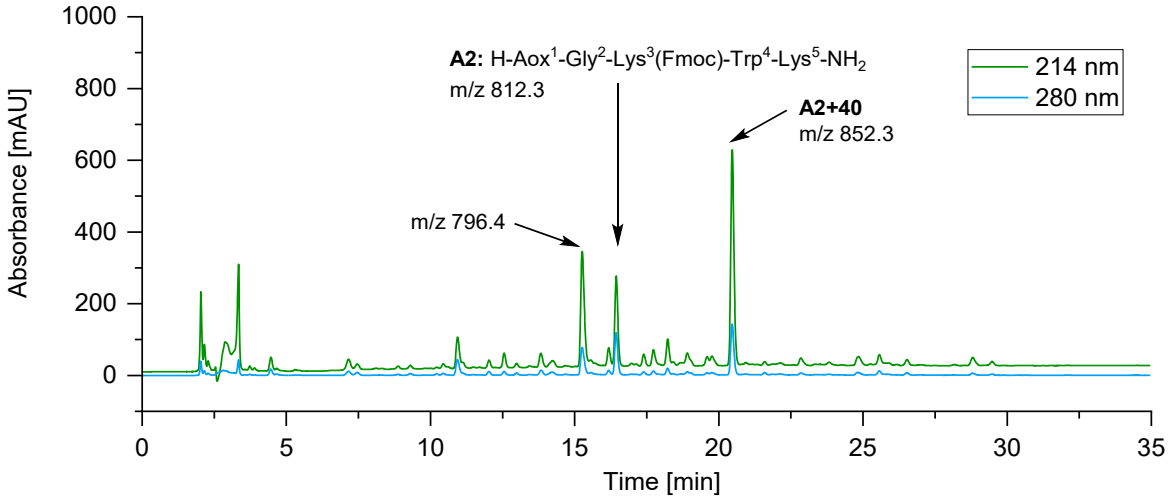


*Fig. 37: HPLC spectra of **A2**. Solvent H_2O + 0.1% TFA. Gradient of 20-65% of B in 30 min.*

After the reaction took place, an HPLC and a MS were performed to verify the purity of the solid obtained. As shown in Fig. 37, the obtained solid was not pure. Two principal byproducts formed. The first is more apolar than **A2** and has a m/z of 796.4 $[M-H]^+$. The second is less polar and has a m/z of 852.3 $[M-H]^+$, which is 40 units more than **A2**, as the byproduct of **A1**.

The purification with a medium pressure flash chromatograph with a C_{18} reversed phase column permitted the separation of the product into two fractions. The first with the pure byproduct with a m/z of 796.4 and the second with the pure **A2** (no **A2+40** was found at first). The purity of the **A2** fraction was confirmed by the MS carried out right after the purification (Fig. 38). Interestingly, the MS of the same vial performed the day after the purification presented the peak of the **A2+40** byproduct.

Both of the purified fractions were stored in the fridge for one night in the form of H_2O/ACN solutions and the MS performed on a new sample of the **A2** fraction also presented the peak of **A2+40,** but in a lower amount with respect to the vial that was kept out of the fridge. This in an ulterior proof that **A2+40** is formed over time in aqueous solutions, its formation is unfavored at lower temperatures and is probably due to the reactivity of the Aox moiety.

In these same conditions also the MS of the first fraction presented a byproduct with a m/z of 40 units more than 796.4. Thus, even though the nature of this 796.4 byproduct is unknown, it probably retains the Aox moiety that then reacts to form the +40 byproduct.

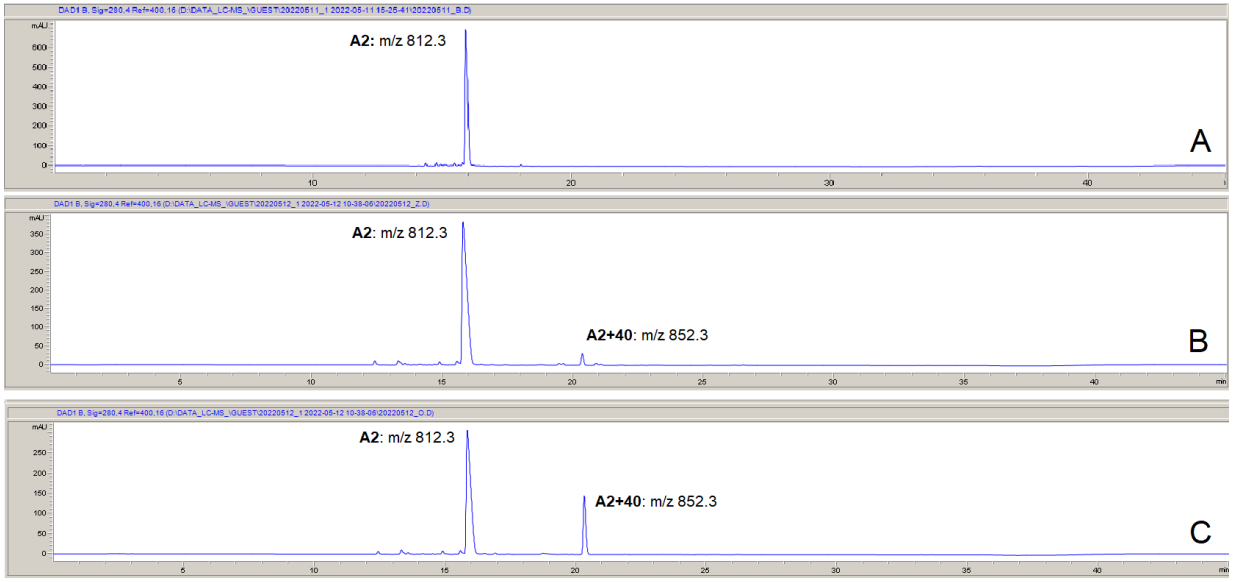


*Fig. 38: LC-MS monitored at 280 nm of the **A2** fraction after the purification, **(A)** Immediately after the purification; **(B)** The day after the purification. Solution stored in the fridge; **(C)** The day after the purification. Solution used for measure A, kept out of the fridge for the night.*

Even more interestingly, the freeze-drying of both fractions determined the almost disappearance of the 796.4 m/z byproduct and **A2**, by a almost complete conversion to their +40 byproducts, respectively.

Peptides C2 and A3

For the sake of convenience, the synthesis from Ile^{14} to Lys^{2} of PMAP-36(12-24) for the consequent production of **C2** and **A3** was carried out using the same batch of resin. Then, the resin was split in two portions and Cys or Aox were attached respectively. Also in this case Boc was used as the side chain protecting group of Lys, Trp, and the N-terminal of Aox; Pbf was used for Arg; and Trt was used for Cys. The TFA treatment was performed in the presence of H_2O and TIS as scavengers for both peptides. In the case of **C2**, also DODT was present to avoid the formation of dimers during the release of the peptide from the resin.

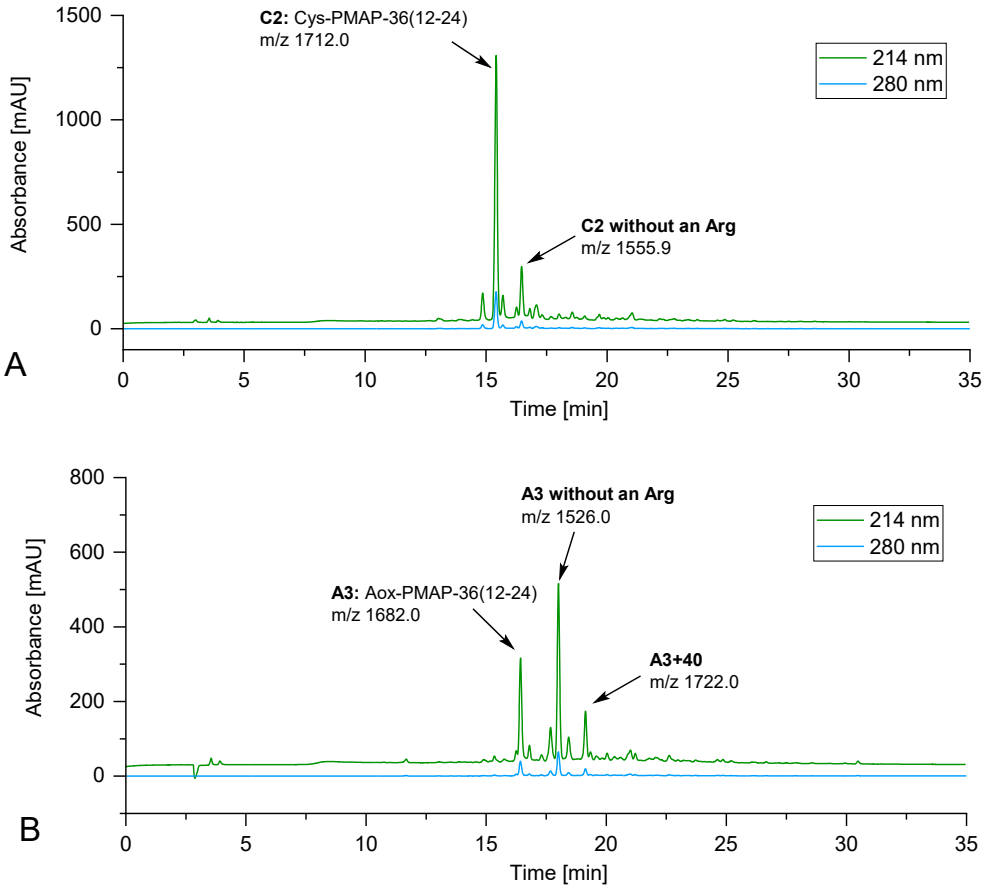


*Fig. 39. HPLC spectra of (**A**) **C2.** Solvent H_2O + 0.1% TFA. (**B**) **A3.** Solvent H_2O. Gradient of 0-50% of B in 30 min for both samples.*

Even though a double Arg coupling was performed, during the synthesis of the PMAP-36(12-24) sequence, the formation of a byproduct without an Arg residue occurred (Fig. 39). In fact, both the HPLC of **C2** and **A3** show a peak with a 156 m/z less than the target peptide and this corresponds to the Arg residue weight. Furthermore, similarly to **A1** and **A2**, **A3** has the less polar **A3+40** byproduct. This also confirms the hypothesis that is the Aox residue reactivity that determines the formation of this type of byproduct. **C2** was further purified through a medium pressure flash chromatography with a C_{18} reversed phase column. This was done to isolate the pure peptide for the future peptide-cotton conjugation. Taken into account, the almost complete conversion of **A2** to its +40 byproduct, the purification of **A3** was postponed until the nature of the +40 byproduct is clarified.

Tests with the +40 byproducts

The +40 byproducts were a shared characteristic of the Aox-terminal peptides. Also, as shown with **A2**, **A2+40** is proven not to form as a direct byproduct of the synthesis but as a consequence of the peptide being in aqueous solution. Therefore, it can be deduced that the formation of this type of byproduct is probably due to the reactivity of the Aox group. Unfortunately, the reactivity of the sole Aox in water could not be assessed due to limitations of the LC-MS instrument that could not detect the mass of this small peptide alone. But other tests were performed trying to determine the nature of the +40 byproducts.

Reaction with Acetone

Acetone is majorly used in the laboratory; furthermore, as a ketone, is reactive towards the Aox group; and determines the formation of a product with a mass 40 units higher than the starting peptide. Thus, even though, the peptides never encounter acetone during the synthesis or the purification, **A1** was dissolved in acetone to test if this some residuals of this solvent could somehow react with the Aox-peptides. However, the HPLC proved that the conjugation of **A1** with acetone produced a different species. In fact, the product formed with acetone has a retention time of 3.1 min, whereas the retention time of **A1+40** is 18.4 min (both measured with a 0-30% of B gradient).

Reaction with K^+

The possible complexation of the potassium ion (MW = 39.1 g/mol) by the Aox- peptides was also hypothesized . To test this, **A1** was dissolved in MilliQ water, and the solution was split in two vials. An excess of KI was added to one of them, and both of them were stirred for one day. Also this test was not successful, in fact no difference in the HPLC spectra could be detected, even after one day.

Vanillin conjugation

At the end, to assess if the +40 byproducts would interfere with the peptide-cotton conjugation, a conjugation test with a carbonyl moiety easy to detect and purchase was performed. The chosen aldehyde was vanillin. To do so, an excess of vanillin was added to a solution of **A1** in a NaOAc/AcOH buffer and HPLC spectra were taken to check the reaction.

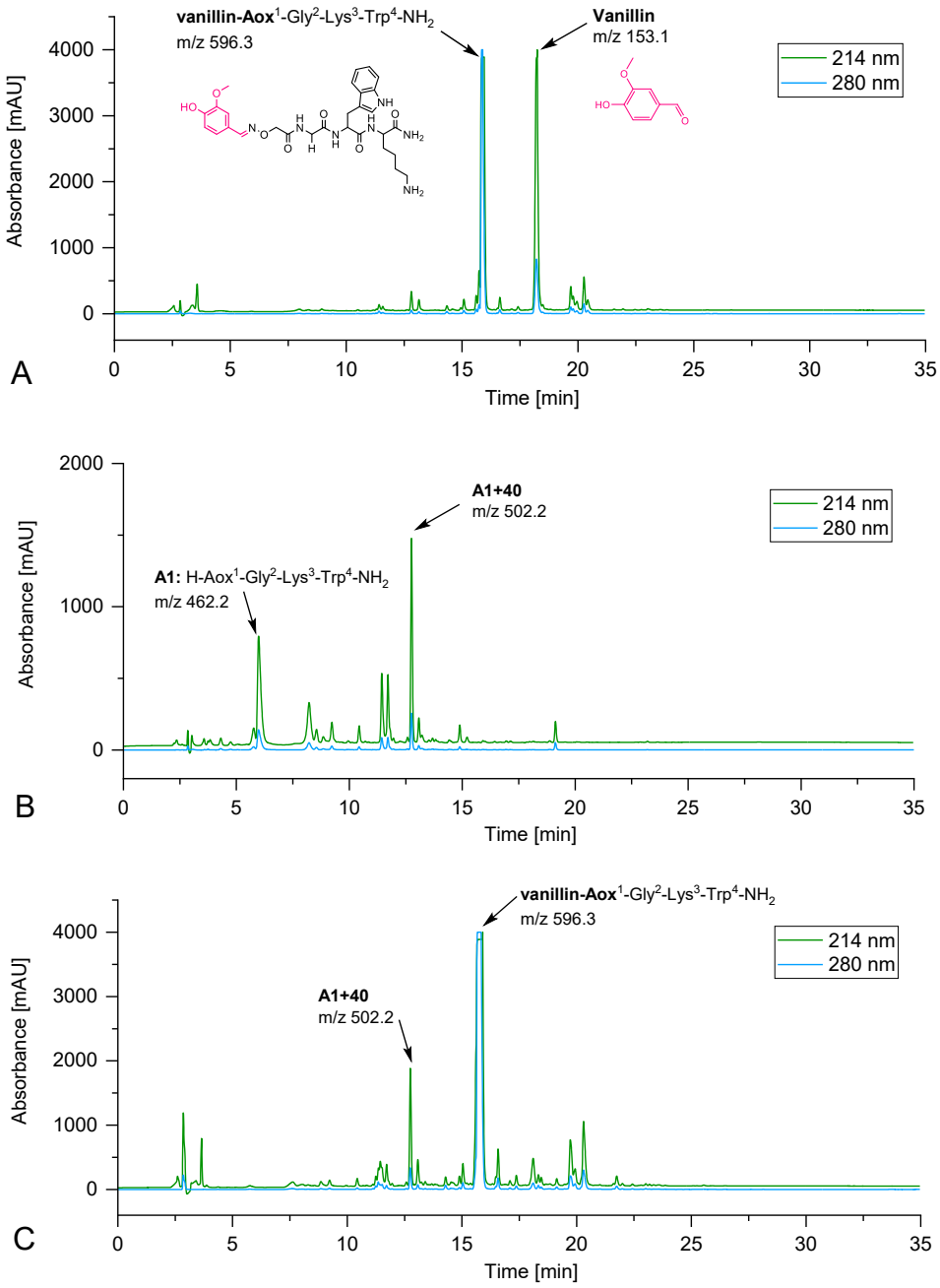


*Fig. 40: HPLC spectra of (**A**) **A1** and vanillin solution in* NaOAc/AcOH buffer. *Chemical structures of vanillin (pink) and the vaillinated product are shown. (**B**) **A1** dissolved in H_2O. (**C**) Co-elution of A and B. Gradient of 0-80% of B in 30 min for all samples.*

As shown in Fig. 40-A, the reaction was successful and the vanillinated peptide was formed. Furthermore, the **A1+40** seems not to be present. Probably this is due to the fact that the Aox reacts preferentially with the vanillin and so the byproduct cannot be formed.

Then, an **A1** aqueous solution (that had already formed the **A1+40**) was coeluted with the vanillin-peptide solution: the peak of **A1** and vanillin disappeared but the one of **A1+40** did not (Fig. 40-B and C). Overall, this demonstrates that the byproduct is not easily formed when the Aox-peptides react with another carbonyl moiety. More importantly, if the byproduct forms, it does not react with the aldehyde moieties present in

solution; and therefore the +40 byproducts do not interfere with the Aox-aldehyde conjugation. This probably suggests that in the +40 byproducts the Aox moiety is occupied or is not present anymore, in fact it does not react.

However, it is still not clear the nature of the +40 byproducts and with whom the Aox-peptides react to form them. Further investigations are necessary in order to use the oxime bond as a convenient linkage strategy for the formation of functionalized cotton materials.

NMR

One-dimensional ^{1}H NMR spectra were recorded at 400 MHz to characterize the probe peptides. Only for peptide **C1**, two-dimensional experiments were also conducted in order to accurately assign all proton chemical shifts and to understand whether this short peptide assumes a known conformation in solution.

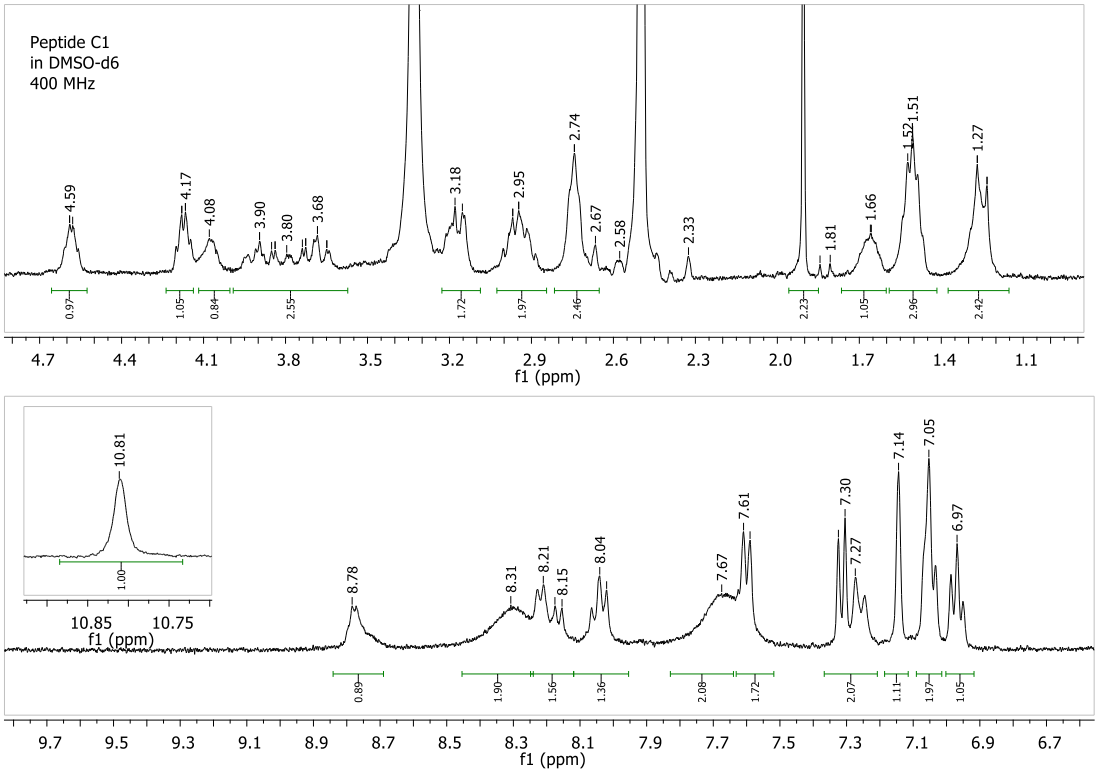


*Fig. 41: ^{1}H NMR one-dimensional spectrum of **C1** (400 MHz, DMSO-d6, 298K).*

For the other peptides one-dimensional spectra allowed to identify and count all protons in the different areas of the spectrum, confirming, together with MS spectra, the correctness of the syntheses. Spectra were obtained by dissolving about 0.5 mg of each peptide in 0.5 ml of DMSO-d6. As an example, the spectrum of **C1** is shown (Fig. 41) in which we distinguish the NH peak of the indole ring of Trp at 10.81 ppm, the NH zone ranging from 8.6 to 6.8 ppm, the αCH zone between 4.6 and 3.5 ppm, and the CH zone of the side chains from

3.2 to 1 ppm. The assignment of the regions of the spectrum just made is similar for all the one-dimensional spectra acquired.

Two-dimensional COSY (COrrelation SpetroscopY), TOCSY (TOtal Correlation SpectroscopY) and NOESY (NOE SpectroscopY) spectra were recorded for peptide **C1**.

COSY and TOCSY are two-dimensional NMR techniques used to correlate the chemical shifts of protons that exhibit J-coupling with each other. These spectra are used to assign all proton frequencies. Through NOESY spectra one goes to map the spatial distance between the protons present. The presence of a cross-peak denotes interaction by nuclear Overhauser effect (or NOE effect) and thus spatial proximity between two protons. Dealing with a peptide chain, one will reasonably observe the interaction by NOE effect of the protons of immediately adjacent residues in the primary structure of the peptide [αCH(i)→ NH(i+1). The NOE effect between protons of non-neighboring residues will be present only if the peptide assumes a secondary structure such that the protons considered are spatially close together.

For **C1** first, the αCH protons of all amino acids in the COSY spectrum and the TOCSY spectrum, the cross peaks of the NH protons of each amino acid, and the protons relative to their own spin system were identified. This allowed the side chains of all amino acids to be completely assigned.

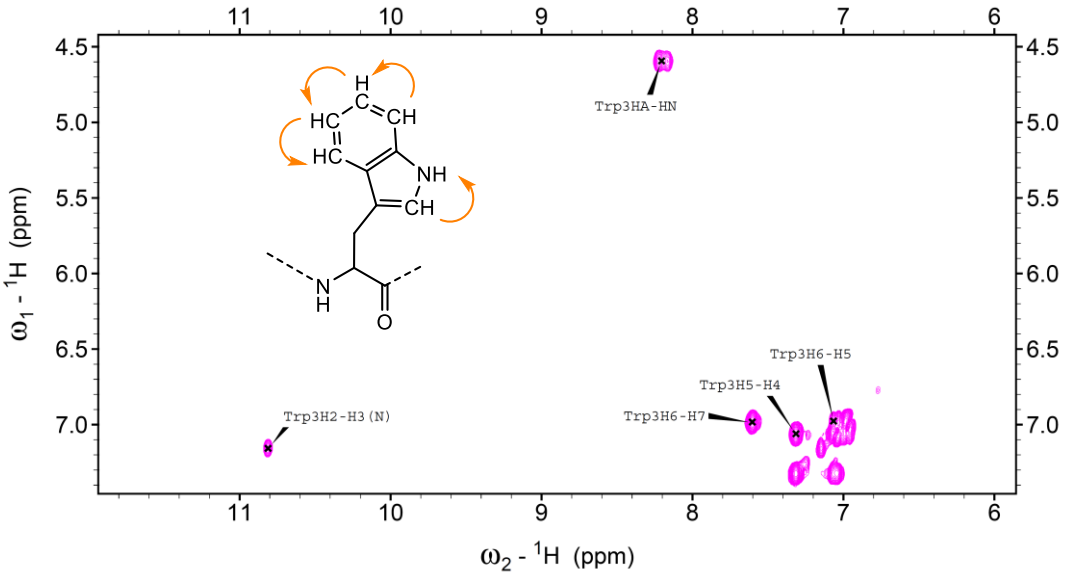


*Fig. 42: Region of the COSY spectrum of **C1** in which the assignment of chemical shifts of the protons of the indole ring of Trp is highlighted (400 MHz, DMSO-d6, 298K)*

NOESY spectrum was needed to assign each NH to a precise amino acid in the peptide sequence. Specifically, sequential assignment is possible by observing the fingerprint zone into which αCH(i)→ NH(i+1) type interactions fall (Fig. 43).

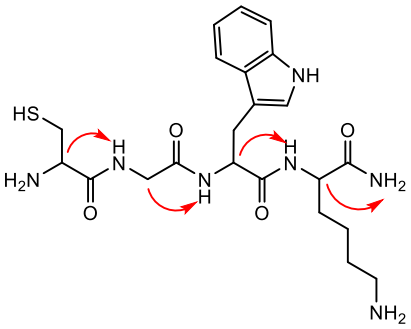


*Fig. 43: Interactions between adjacent protons, of the type αCH(i)→ NH(i+1) in the primary structure of **C1***

They refer to protons of neighboring amino acids in the sequence; in particular, the following are observed αCH Cys[1] →NH Gly[2], αCH Gly[2]→NH Trp[3], αCH Trp[3]→NH Lys[4] (Fig. 44).

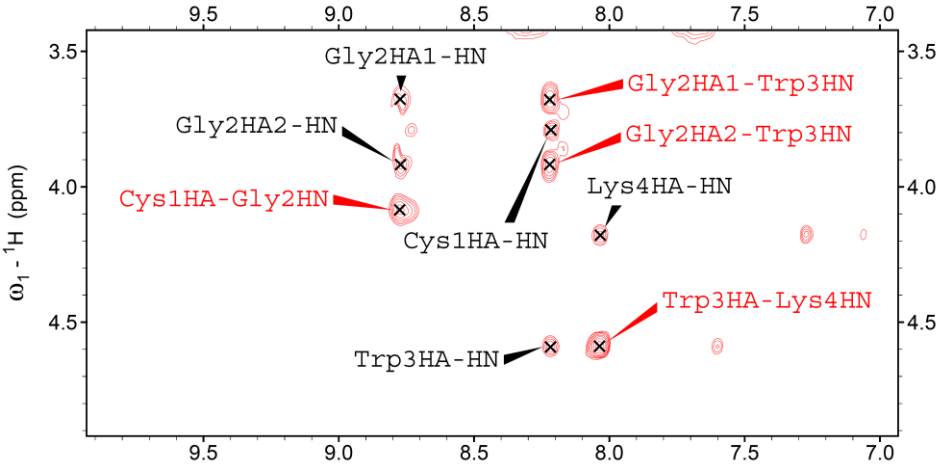
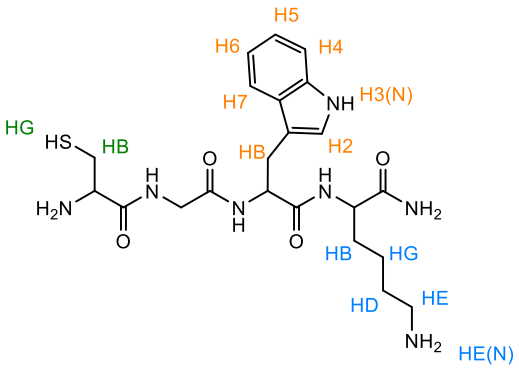


Fig. 44: Fingerprint region of the NOESY spectrum of peptide C1 (400 MHz, DMSO-d6, 298K). In red are the αCH(i)→ NH(i+1) interactions.

The complete assignment of proton resonances of **C1** is shown in the table below:



	HA	HB	HD	HE	HE (N)	HG	HN
Cys[1]	4.011	2.967 3.201	-	-	-	-	8.216
Gly[2]	3.682 3.913	-	-	-	-	-	8.779
Trp[3]	4.588	2.947 3.164	-	-	-	-	8.208
Lys[4]	4.173	1.548	1.505	2.745	7.637	1.265 1.664	8.037
	H2	**H3 (N)**	**H4**	**H5**	**H6**	**H7**	
Trp[3]	7.156	10.81	7.316	7.063	6.980	7.603	

*Table 6: Chemical shifts of **C1***

From the NMR study, we could not identify an ordered conformation for the peptide since there are no distant interactions present in the spectrum to confirm this hypothesis. Therefore, we confirm a disordered conformation for **C1**.

3.6 PEPTIDE-COTTON CONJUGATION

Once the groups that are selective towards the reaction with aldehyde are introduced into the peptide chain, the functionalization of the oxidized cotton can take place. Two types of chemoselective ligation were selected for this purpose: thiazolidine and oxime. The first involves a β-amino thiol and an aldehyde, whereas the second involves and alkoxyamine and a carbonyl moiety (see paragraph 2.2). In this thesis, the aldehyde is generated at the C_6 atom of the glucose when cotton fibers are oxidized, whereas the thiol and the alkoxyamine were introduced as Cys and Aox at the N-terminal of the peptides chains, respectively.

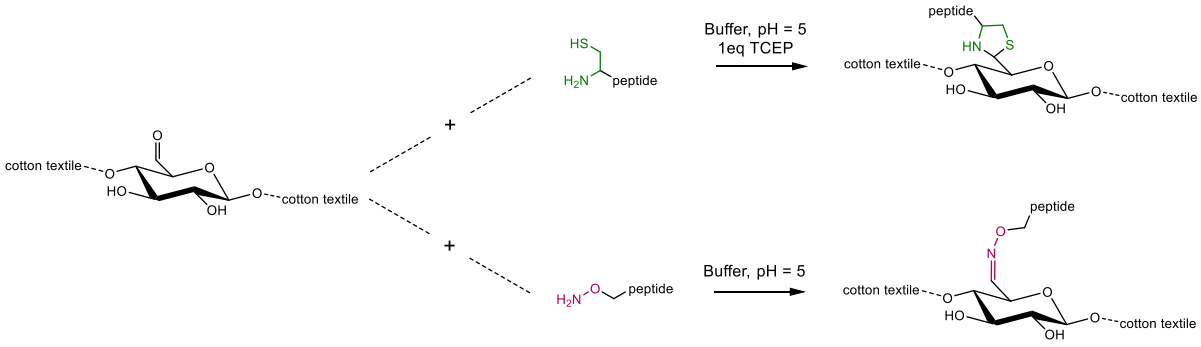


Fig. 45: Thiazolidine and oxime bond formation.

Calculation of the peptide loading by Trp absorbance

All the peptides used for the conjugation with cotton have a Trp in their chain. This allows the reaction to be monitored through UV-vis. In fact, Trp absorbs at 280 nm with an extinction coefficient of 5630 M^{-1} cm^{-1}[91], thus at the beginning of the reaction between the peptide and the aldehyde (when all the peptide is in solution), the concentration of peptide in solution is at its maximum, and so is the Trp absorbance. As the reaction proceed, the cotton "captures" some of the peptide (Fig. 46). Therefore, the concentration of peptide in solution and the absorbance of Trp decrease. The reactions were considered complete when there was no more decrease in the Trp absorbance, and thus the saturation of the cotton was reached.

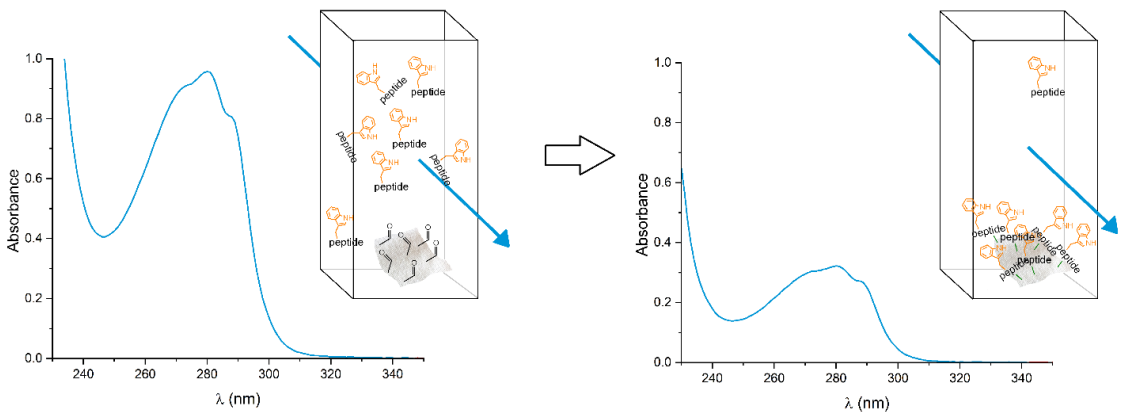


Fig. 46: Schematic representation of the use of UV-vis to monitor the reaction and evaluate the peptide loading on cotton. Trp shown in orange.

The difference between the final and initial absorbance at 280 nm allows the determination of the moles of peptide bound to cotton. And the ratio of mmol of bound peptide and grams of cotton is the peptide loading. In fact:

- The Lambert-Beer law permits to calculate the initial and final concentration of Trp (and thus of the peptide) in solution as $C = \frac{A}{\varepsilon \cdot l}$; where C is the concentration, A the absorbance, ε the extinction coefficient and l the optical path.
- Considering $\Delta C = C_i - C_f$; where C_i is the concentration of peptide in solution before the addition of cotton and C_f is the concentration of the last measure.
- $n_{bound-peptide} = \Delta C \cdot V$; where V is the volume of the solution used for the reaction.
- The **peptide loading** is calculated as $\frac{n_{bound-peptide}\,(mmol)}{m_{Cotton}\,(g)}$ and quantifies the amount of peptide bound to the cotton. Ideally this would also correspond also to the total mmoles of aldehyde sites on cotton.

Thiazolidine bond formation

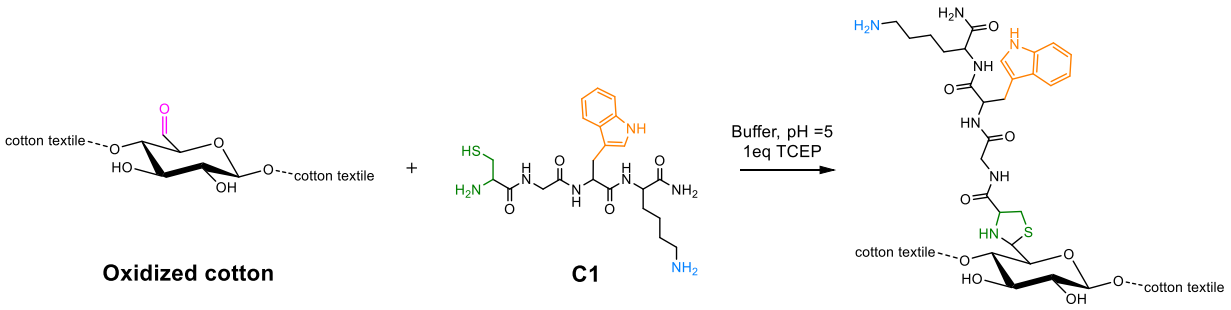


*Fig. 47: Thiazolidine bond formation with **C1**.*

The aldehydes present on the cotton surface do not have a vicinal C-terminal ester. Thus, the final intramolecular O- to N-acyl transfer described in paragraph 2.2 does not take place and the reaction stops with the formation of the heterocyclic ring. The reaction takes place in an aqueous solution at a slightly acidic pH to avoid the reaction of the aldehyde with other nucleophiles present in the peptide. The presence of and reducing agent (TCEP) is needed to prevent the formation of the peptide dimer through the disulfide bond between Cys of two peptide molecules. Moreover, given the high chemoselectivity of this reaction, the peptide reacts without the need of protecting groups.

C1 was used to test the thiazolidine ligation. In particular:

- A-, B- and C-cotton samples were compared. They were subjected to the same procedure to quantitatively evaluate if they presented a different peptide loading and consequently a different degree of oxidation.
- The relationship between reaction time and peptide concentration was investigated.

- The possibility of reusing the oxidation mixture was examined. The peptide loading of A-cotton oxidized during the 1st, 2nd and 3rd use were compared to roughly evaluate the different degree of oxidation that was already observed qualitatively upon the Schiff test treatment.
- The possibility of reusing the peptide solution was tested.

A-, B- C-cotton comparison

In the first experiments a theoretical loading of 0.02 mmol/g was taken in consideration to set the parameters for the reaction. In fact, previous experiments[6] seemed to prove the maximum peptide loading to be around that value.

Thus, the following parameters were chosen to test all three cottons (EXP-1):

- The actual peptide-cotton ratio used for A- and B-cotton was 0.01 mmol/g was used because the solid obtained from the synthesis of **C1** probably contained some salts and thus the weighted solid did not correspond to the actual amount of peptide present in solution. The actual concentration was established from the absorbance of the peptide solution before the addition of the oxidized cotton. With C-cotton, this error was taken in consideration and the obtained peptide-cotton ratio was 0.04 mmol/g. In this way the peptide was thought to be in a slight excess with respect to the aldehyde sites on cotton.
- The concentration of the peptide in solution was around 0.15 mM. In this way the reaction could take place in a cuvette and be directly monitored by UV-vis without errors derived from the transfer or dilution of solution. In fact, a concentration lower than 0.17 mM determines an absorbance lower than 1 and, thus in the range of validity of the Lambert-Beer law.
- The 3 mL cuvette remained closed for the whole process to avoid a loss of solution by evaporation.
- 1eq of TCEP, with respect to the peptide, was added to ensure that the peptide did not form the dimer.

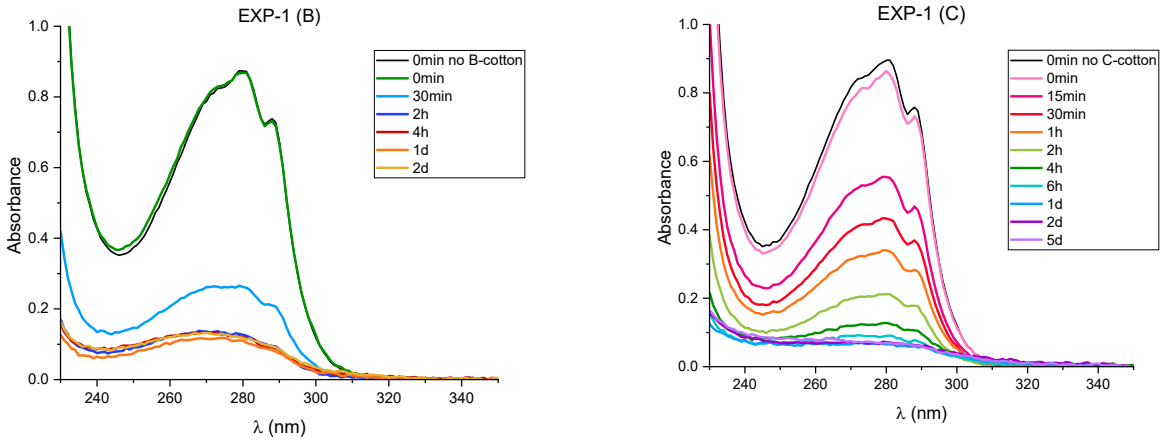


Fig. 48: UV-vis spectra of EXP-1 with cotton B and C.

The reaction with each of the three cottons resulted successful. In particular, a decrease in the absorbance of the Trp could be detected. The saturation point was reached very quickly for A- and B-cotton, in fact in less than 2 hours, whereas the saturation of C-cotton required 1 day. This is probably due to the higher peptide-cotton ratio of C. Even so, in all cases, all the peptide present in solution is "captured" by cotton, in fact the absorbance is almost nullified in all the reactions (Fig. 48). To confirm that the peptide is effectively bound to cotton, Kaiser test were performed, and they all resulted positive. But to see if there were still some free aldehydes sites on the cotton, Schiff tests were performed and for all three cottons they resulted slightly pink. This suggested that the peptide present in solution was in defect with respect to the aldehyde sites. Thus, the obtained peptide loading is fictitious and just corresponds to the peptide present in solution (Table 7).

Therefore, another experiment was set with some changes in the procedure (EXP-2):

- A concentration less than 0.17 mM allows the reaction to be monitored directly without the need of dilution to stay in the absorbance zone where the Lambert-Beer law is still valid. Thus, the peptide concentration remained the same, but a higher volume was used. In this way, the peptide was present in an excess with respect to the aldehyde sites on cotton and the number of aldehyde sites that reacted was maximized. Therefore, a valid peptide loading could be calculated.
- The reactions took place in a 50 mL flask and thus to perform the UV-vis measure 3 mL of solution were removed from the reaction and they were put back in the reaction solution at the end of the measure.

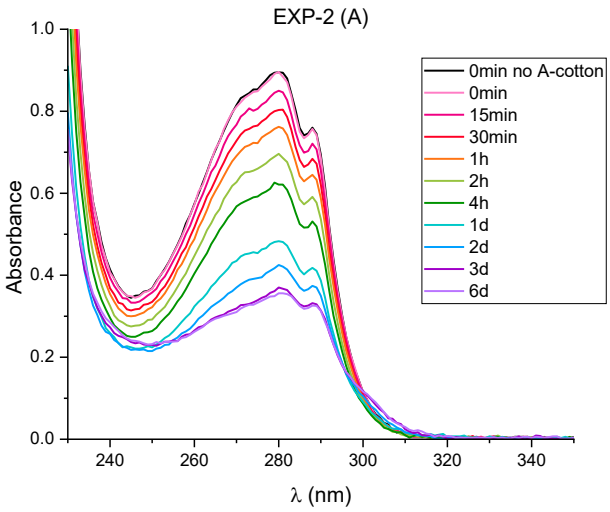


Fig. 49: UV-vis spectra of EXP-2 with cotton A.

Also in this case all cottons reacted successfully with the peptide, in fact in all cases a decrease in the Trp absorbance could be appreciated. This is confirmed by the positive Kaiser tests. Furthermore, as shown in the example of Fig. 49, with these reaction conditions a saturation point was reached when there was still some peptide in solution (the final absorbance is much higher than zero). This suggests that the number aldehyde

sites that reacted with the peptide to form a thiazolidine bond was maximized. This was verified by the absence of color change upon the Schiff reactant addition to the functionalized cotton pieces. The higher peptide-cotton ratio determined a longer reaction time of 3 to 6 days (Table 7).

Sample	Peptide-cotton ratio (mmol/g)	Loading (mmol/g)	Time
EXP-1 (A)	0.0119	0.010	2 hours
EXP-1 (B)	0.0131	0.011	2 hours
EXP-1 (C)	0.042	0.040	1 day
EXP-2 (A)	0.436	0.162	3 days
EXP-2 (B)	0.632	0.150	3-5 days
EXP-2 (C)	0.626	0.145	3-5 days

Table 7: Summary table for EXP-1 and EXP-2. The wording "3-5 days" means that the reaction was monitored at the 3rd and 5th day and that at the 3rd day the reaction was not completed yet whereas at 5th day it was for sure. But there is an uncertainty about when it completed between the 3rd and the 5th day because there are no data in between.

In terms of peptide loading and reaction times no differences were noticed between the three types of cotton. Indeed, all required 3-5 days to complete the reaction and determine a loading around 0.15 mmol/g. The obtained loading is surprisingly much higher that the theoretical one.

However, the different cottons differ in the stability of the cotton texture: C-cotton tends to disintegrate releasing small pieces of cotton in the solution; the wide weft of A-cotton tends to fall apart in single threads and after more than 6 days of reaction acts as C-cotton; on the other hand, B-cotton slightly releases some small pieces of tissue in solution only after more than 5 days of reaction. Therefore C-cotton was not used anymore, and B-cotton was chosen as the preferred one for future experiments.

Role of concentration

The role of concentration was clarified by EXP-3 and EXP-4:

- In the first the conjugation of A-cotton with **C1** was performed using a peptide concentration of 2 mM, whereas in the secondo B-cotton and **C1** reacted in a peptide solution of 1 mM.
- To monitor the reaction of EXP-3, 80 µL were withdrawn and diluted at each measure. This volume was wasted in order not to modify the concentration of peptide in solution.
- From EXP-4 on, cuvettes with an optical path of 1mm were chosen. In this way the measure could be performed without dilution and the solution could be put back in the reaction mixture avoiding both dilution and volume errors that were characteristic of EXP-3.
- A higher peptide-cotton ratio was present. In this way, there was still peptide in solution that could be re-used for the conjugation with other pieces of cotton (see the next paragraph).

Sample	Peptide-cotton ratio (mmol/g)	Loading (mmol/g)	Concentration (mM)	Time
EXP-3 (A)	0.987	0.160	2.18	1 day
EXP- 4 (B)	1.21	0.100	1.05	1 day

Table 8: Summary table for EXP-3 and EXP-4.

As summarized in Table 8, a higher concentration did not impact the peptide loading but seemed to decrease the time of reaction. This was further confirmed by the next experiments (see next paragraph). Therefore, to maximize the number of experiments a concentration of 1-2 mM was chosen as a standard. The value of the peptide loading of EXP-3 is the same obtained in EXP- 2(A). On the other hand, the peptide loading with B-cotton is slightly lower than in EXP-2(B). The reason for this discrepancy is not clear. Also in this case the Schiff and Kaiser verified that the reactions effectively took place completely (ex. In Fig.50). In fact, in both cases, the Kaiser tests resulted blue, whereas the cottons subjected to the Schiff test did not change color.

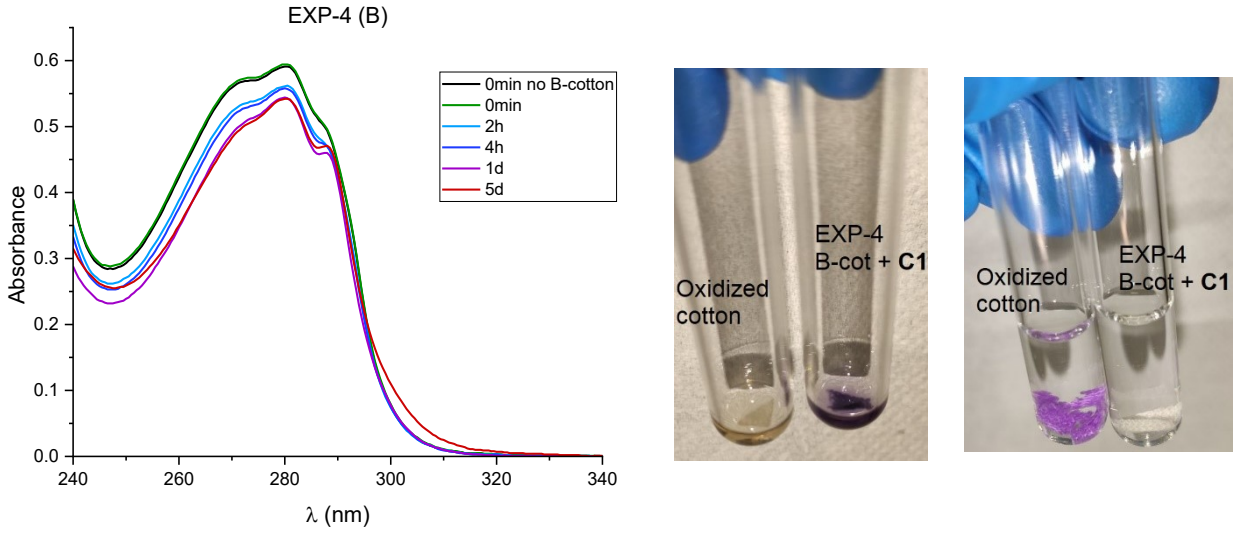


*Fig. 50: **On the left**: UV-vis spectra of the EXP-4 reaction. **In the center**: Kaiser test; **On the right**: Schiff test on the functionalized cotton of EXP-4*

Re-use of the peptide solution and peptide excess

Considering the desirable industrialization of this type of process, the potential re-use of the peptide batch is attractive. Thus, this possibility was explored with EXP-6 and EXP-7.

In EXP-6 a known volume of the peptide solution used in EXP-3 was used to functionalize another piece of oxidized A-cotton. Before the new reaction, the solution was filtered to eliminate all the possible residues of cotton from the previous conjugation.

In EXP-7 the same procedure of EXP-6 was performed, but with the oxidized B-cotton and the peptide solution of EXP-4. Furthermore, at the beginning of the new reaction 1eq of TCEP, with respect to the peptide, was added to the solution to make sure that all the peptide present in solution was not in the dimeric form. In this case the procedure was repeated three times (each time 1 eq of TCEP was added). Between consecutive re-uses the solution was stored in the fridge.

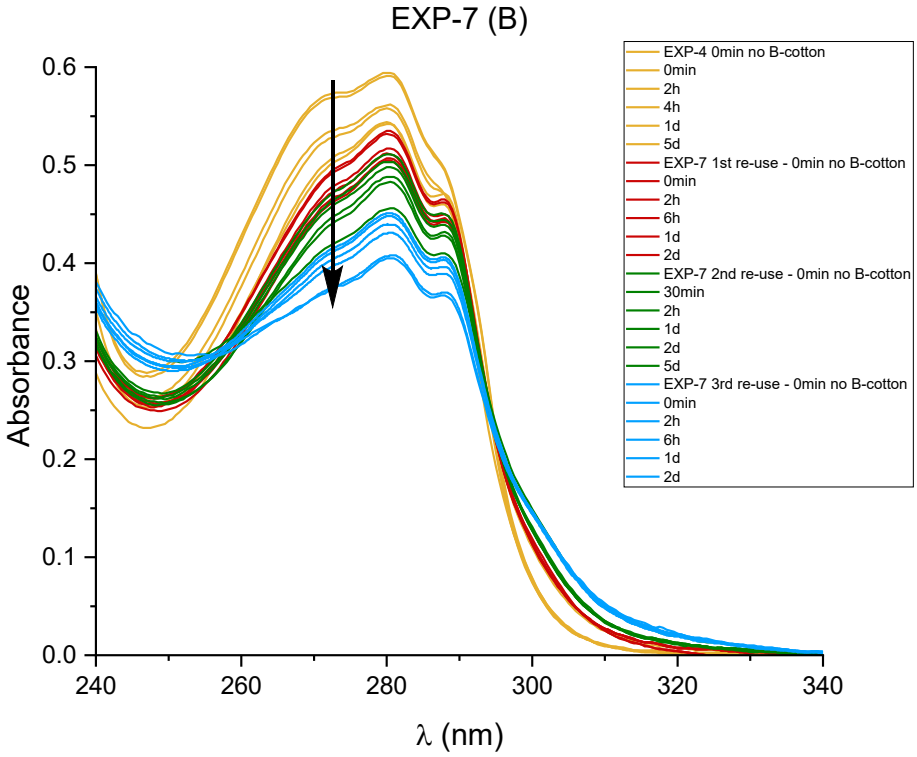


Fig. 51: UV-vis spectra of EXP-4 and EXP-7. A different color means a different piece of oxidized B-cotton. In yellow all the spectra of EXP-4. In red EXP-7 1st re-use. In green ECP-7 2nd re-use. In blue EXP.7 3rd re-use.

Sample	Peptide-cotton ratio (mmol/g)	Loading (mmol/g)	Concentration (mM)	Time
EXP-6 (A)	1.93	0.283	1.84	1 day
EXP-7 1st re-use (B)	2.00	0.105	0.950	
EXP-7 2nd re-use (B)	1.94	0.213	0.908	
EXP-7 3rd re-use (B)	1.95	0.187	0.796	

Table 9: Summary table for EXP-6 and EXP-7

The re-use of the solution did not affect the conjugation between Cys and aldehyde. In fact, both in EXP-6 and EXP-7 a decrease in the absorbance of Trp could be appreciated. This further confirms that, in the experiments after EXP-1, the decrease of the absorbance stops due to the saturation of the aldehyde sites of cotton with the

peptide and not due to the defect of peptide in solution. Furthermore, all the re-uses required 1 day, as the experiments with the fresh peptide solution. Interesting to note is the fact that at the 2nd re-use in EXP-7 the color of the solution turned from colorless to slightly yellow. An HPLC was performed but there were no difference with respect to the first re-use and no dimer was present in solution.

The peptide loading results are not so clear to interpret. In fact, in EXP-6 and in the 1st re-use of EXP-7 the used peptide-cotton ratio was the double with respect to EXP-3 and EXP-4. This was done to test if a higher excess of peptide could drive the reaction to the thiazolidine bond formation or if it had a role in the reaction time. The reaction time was not shortened. Thus, it is confirmed the previously speculated role of the concentration with regard to the reaction time. The peptide loading obtained in EXP-6 is almost the double of the one obtained in EXP-3. Therefore, it seemed to demonstrate that a higher peptide excess drives the reaction towards the thiazolidine formation and in this way a higher peptide loading can be obtained. On the other hand, the higher peptide excess in EXP-7-1st did not impact the value of the loading. But in the 2nd and 3rd re-use the loading was indeed almost the double of what it was obtained in EXP-4. In all case, the Kaiser tests resulted blue, whereas the cottons subjected to the Schiff test did not change color. A peptide-cotton ratio of 1-2 mmol/g were chosen as a standard for future experiments.

Addition of TCEP

The solution used for EXP-7 was utilized one last time to see the effect of an excess of TCEP addition (EXP-9, Fig. 52). At first, the cotton was added without the additional TCEP. The amount of added cotton was enough to obtain a peptide-cotton ratio of 2.3 mmol/g and the concentration of peptide in solution was 0.725 mM.

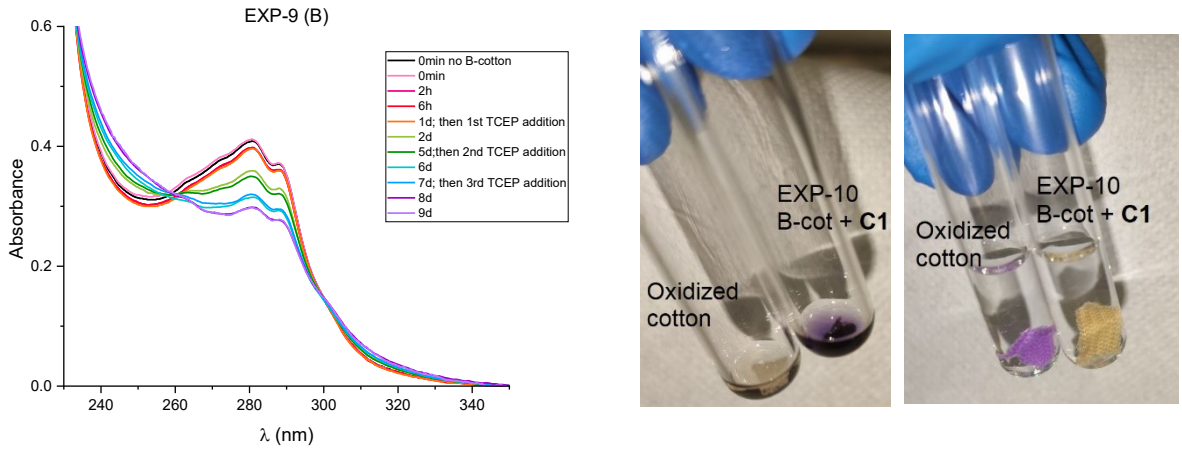


Fig. 52: ***On the left****: UV-vis spectra of the EXP-10 reaction.* ***In the center****: Kaiser test;* ***On the right****: Schiff test on the functionalized cotton of EXP-10*

Sample	TCEP	Loading (mmol/g)
EXP-9 (B)	-	0.073
	First addition	0.355
	Second addition	0.499
	Third addition	0.620

Table 10: Summary table for EXP-9

After 6h the reaction was already finished with a peptide loading of 0.073. Then 1 eq of TCEP, with respect to the peptide in solution, was added. The absorbance at 280 nm dropped and a loading of 0.355 mmol/g was obtained. This procedure was repeated for other two times and the final peptide loading was of 0.620 mmol/g (Table 10 and Fig. 52). It is important to notice that the shape of the UV-vis spectra changed over time and the presence of two isosbestic points suggest the presence of an equilibrium of three species. TCEP is a reducing agent with a redox potential of -0.29 V[93]. It is introduced in solution to avoid the formation of a disulfide bond, but when present in an excess could probably reduce also some other moieties present in solution. Therefore, considering the unusual conditions of reaction, this experiment was not taken into consideration for the peptide loading evaluation. As shown in Fig. 52, upon this treatment the cotton assumed a yellow shade that was not removable either with water or acetone washings. This could be an ulterior suggestion that the solution degrades over time and that something formed in solution, that is not the original peptide **C1**, was linked to the cotton. However, the presence of the amine moiety on cotton was confirmed by the blue Kaiser test, whereas the absence of free aldehydes sites was verified by the Schiff test (Fig. 52). Still, the conditions of this experiment did not allow conclusive results and further investigations are needed.

Re-use of the oxidation mixture

To test the hypothesis that the oxidizing mixture of TEMPO/laccase loses its oxidating power over time and multiple uses (paragraph 4.2), EXP-10 was designed:

- A fresh solution of **C1** in a concentration of 1.07 mM was prepared and 1 eq of TCEP was added
- The solution was split in three vials with the same known volumes.
- A-cotton obtained from different re-uses of the oxidation mixture was added to each vial with similar peptide-cotton ratios and the absorbance of each solution was monitored for two days. All three reaction were complete after one day (Table 11).

Sample	Peptide-cotton ratio (mmol/g)	Loading (mmol/g)
EXP-10 - 1st ox (A)	1.36	0.147
EXP-10 – 2nd ox (A)	1.53	0.117
EXP-10 – 3rd ox (A)	1.34	0.069

Table 11: Summary table for EXP-10

As summarized in Table 11, the peptide loading obtained with the 1st-oxidation cotton is coherent with the previous measurements; the one obtained with the 2nd-oxidation cotton is slightly lower; and the last one is half the value of the first. This is probably due to a lower amount of aldehyde sites on the different cotton pieces, and it is a confirmation of what was observed upon Schiff reagent addition.

Oxime bond formation

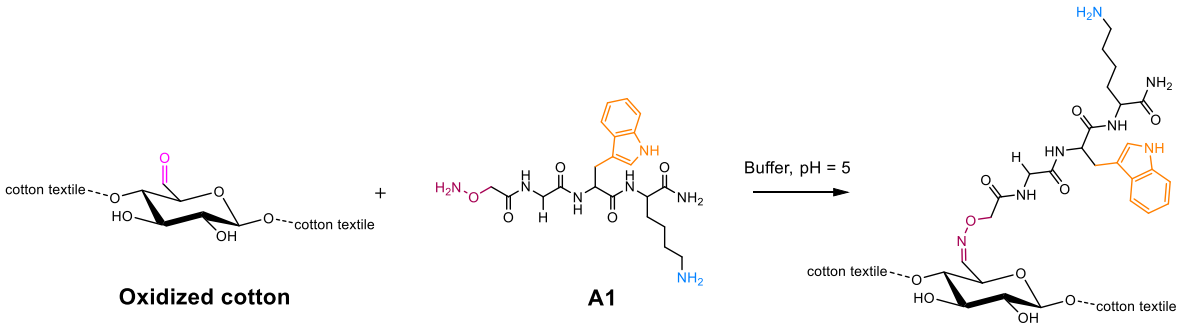


*Fig. 53: Oxime bond formation with **A1***

Similarly to the thiazolidine ligation, the reaction forming an oxime takes place in an aqueous solution at a slightly acidic pH. In this case, there is no need to add TCEP because Aox does not dimerize. Since it was demonstrated that the +40 byproduct of Aox-ending peptides does not interfere with the ligation, **A1** was used to compare the oxime ligation with the thiazolidine one. In particular:

- The optimized conditions of reaction used found with **C1** were tested to see if there was any difference in the peptide loading (EXP-5)
- The possibility of re-use of the peptide solution was explored (EXP-8)

The used reaction conditions were similar to the ones chosen for **C1**. In particular, **A1** was dissolved in solution in a concentration of 1.7 mM and the amount of added cotton determined a peptide-cotton ratio of 2.04 mmol/g. (EXP-5).

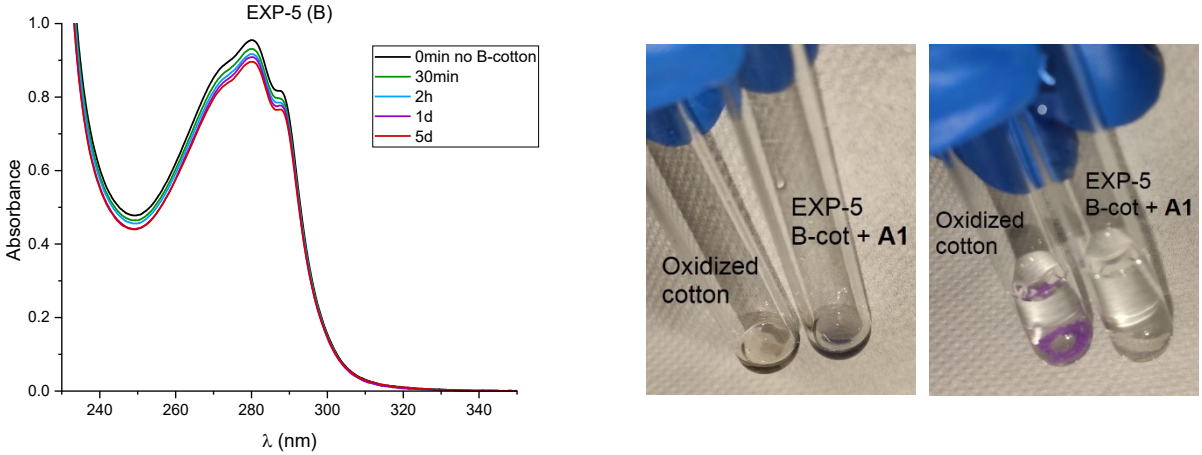


*Fig. 54: **On the left**: UV-vis spectra of the EXP-5 reaction. **In the center**: Kaiser test; **On the right**: Schiff test on the functionalized cotton of EXP-5.*

The reaction of Aox and the aldehydes on cotton resulted successful. Similarly to the experiments with **C1**, a decrease in the absorbance of the Trp could be detected. This suggest that the reaction actually took place, as confirmed by the blue Kaiser test and the absence of color change upon the Schiff reactant addition (Fig. 54). Furthermore, the reaction time of 1 day and the peptide loading of 0.126 are comparable to what was obtained in the case of the thiazolidine bond.

When the EXP-5 reaction was complete, the functionalized cotton was removed from the solution and another oxidized piece of cotton was added. This was done for two times (EXP-8). The peptide-cotton ratio was maintained around 2 mmol/g in all the repetitions (Table 12).

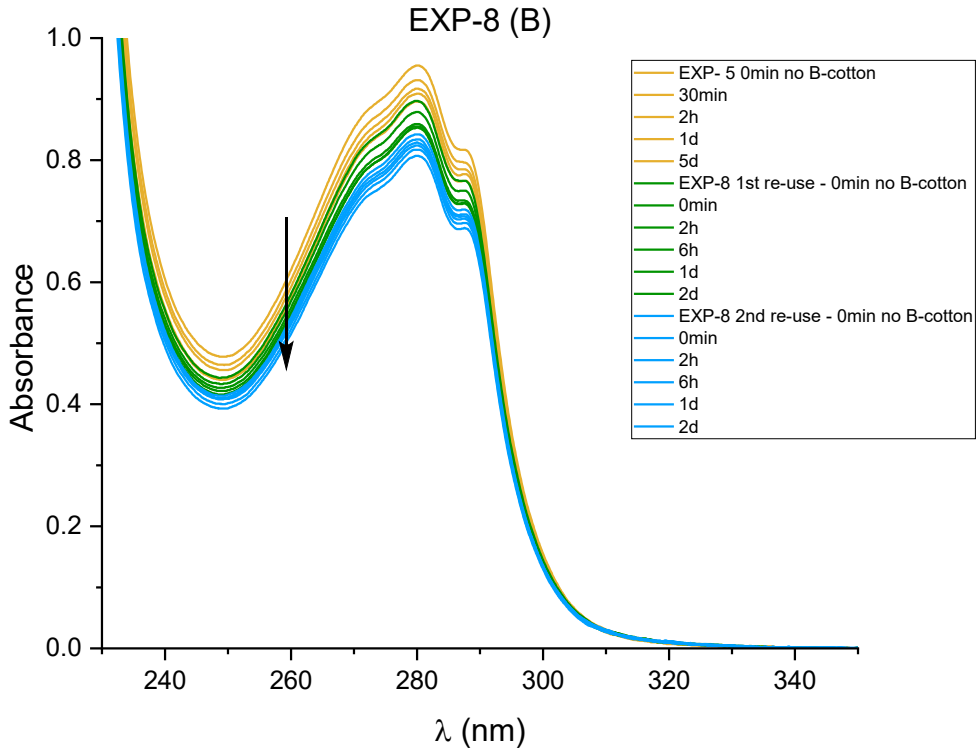


Fig. 55: UV-vis spectra of EXP-5 and EXP-8. A different color means a different piece of oxidized B-cotton. In yellow all the spectra of EXP-5. In green ECP-8 1st re-use. In blue EXP-7 2nd re-use.

Sample	Peptide-cotton ratio (mmol/g)	Loading (mmol/g)	Concentration (mM)	Time
EXP-5 (B)	2.04	0.126	1.70	1day
EXP-8 1st re-use (B)	1.90	0.093	1.59	
EXP-8 2nd re-use (B)	2.16	0.090	1.50	

Table 12: Summary table for EXP-5 and EXP-8

As shown in Fig. 55 and table 12, the re-use of the solution did not impact the ligation of Aox with the oxidized cotton. The success of the reaction was also confirmed by the positive Kaiser test and negative Schiff test. The reaction time remained the same, but a slight decrease in the peptide loading upon recycling could be appreciated. Considering that the peptide-cotton ratio is similar to EXP-5, this result is unexpected. Further investigations are needed in this instance, for example using a much lower concentration with the same peptide-cotton ratio.

It is also important to notice that when Fig. 51 and Fig. 55 are compared, the degradation of the peptide solution observed with **C1** is not detected with **A1**. Maybe this is due to the absence of the TCEP phenomenon.

Comparison of Thiazolidine and Oxime ligations

The amino acidic chain of **C1** and **A1** allows an optimal comparison of the two investigated chemoselective ligations with oxidized cotton. In fact, they only differ for the N-terminal amino acid. Thus, the most relevant characteristics of the ligation are summarized and compared below:

Reactivity of the chemoselective group

Even though both Cys and Aox are selective towards the reaction with aldehydes, they both present side reactions. In an aqueous solvent, the thiol group of Cys is reduced by another thiol to form disulfide bonds. Whereas the Aox moiety in all the synthetized peptides react to form the +40 byproduct. Both these side-reaction are manageable and do not affect much the outcome of the conjugation: the disulfide bond is easily broken by the presence of a reducing agent (in this case TCEP), whereas the formation of the +40 byproduct is inhibited by the presence of aldehydes and when it forms it does not react with aldehydes.

Reaction conditions

In the same solvent and pH, the reaction time and the concentration required to react with the oxidized cotton are the same. But **C1**, to prevent the formation of dimers through the disulfide bond, needs also the additional presence of TCEP in solution.

Outcome of the reaction

Both ligations were successful, and the UV-vis monitoring allowed the quantification of the peptide loading with both ligations. Overall, the peptide loading obtained with fresh solutions of both **C1** and **A1** seems to be similar and around 0.15 mmol/g. This suggest that both Cys and Aox are able to react with almost all the present aldehyde sites and saturate the cotton sample.

Stability and re-utilization of the peptide solution

Over time the **C1** solution seems to degrade, whereas **A1** seems not to. This is already appreciable when the last and second-to-last measurement of EXP-4 and EXP-5 are compared. In fact, in both experiments a fresh peptide solution was used to conjugate to cotton, and they were both monitored for 5 days. In EXP-4 the last

spectrum changes with respect to the second-to-last (it becomes slightly broader), whereas in EXP-5 this does not happen (Fig. 56 A and B). This phenomenon is more visible when the first and third uses of the same solution are compared (Fig. 56 C and D). In fact, the shape of **A1** solution remains the same whereas the peak of the spectrum of the **C1** solution becomes broader over time. This type of degradation could be due to the presence of TCEP. In fact, in the previous paragraphs it was shown how the **C1** spectrum changes upon an excess of TCEP addition.

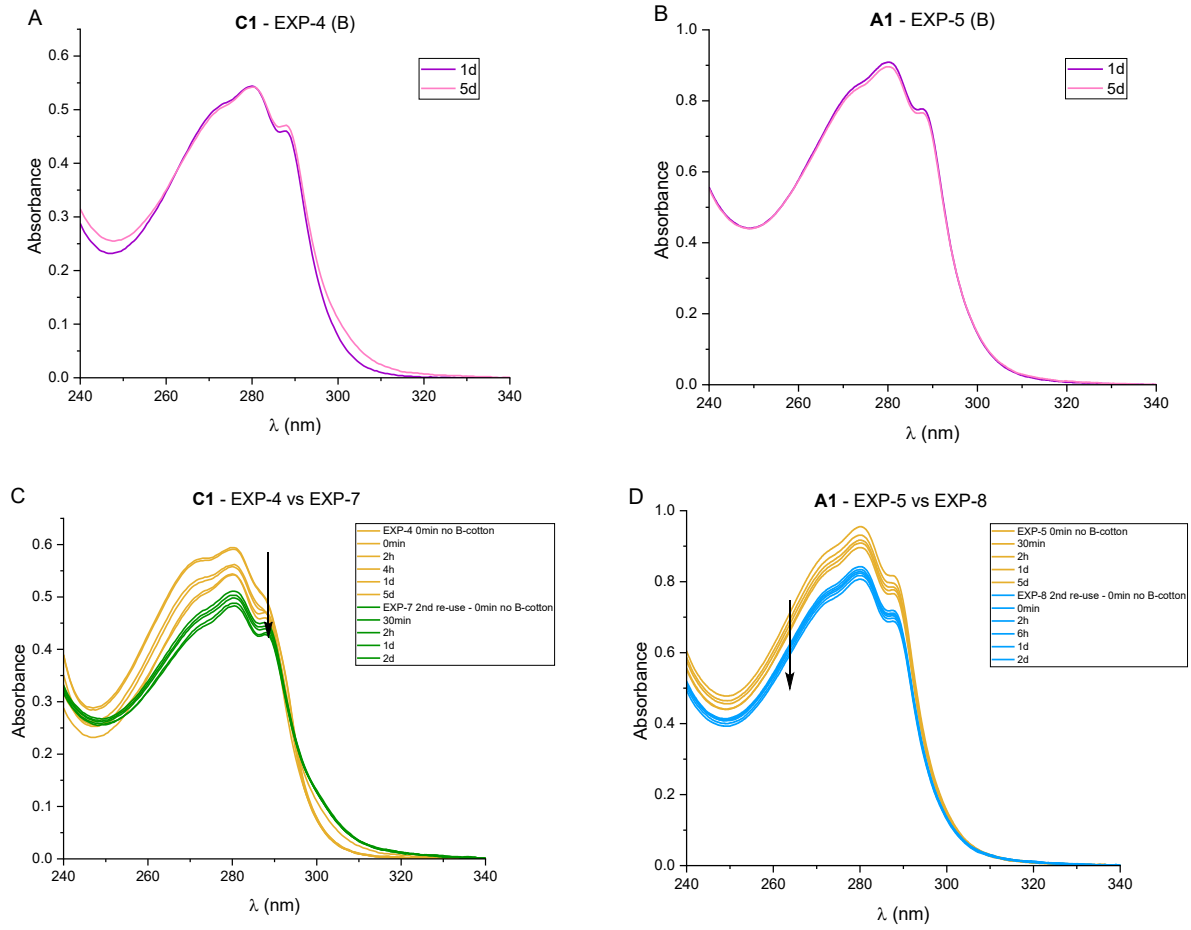


*Fig. 56:UV-vis spectra of (**A**) EXP-4 at 1 day and 5 days ; (**B**) EXP-5 at 1 day and 5 days; (**C**) first use and third use of **C1** solution for the conjugation with cotton (EXP-4 and EXP-7) ; (**D**) first use and third use of **A1** solution for the conjugation with cotton (EXP-5 and EXP-8).*

Overall, thiazolidine and oxime ligation takes place in similar conditions and, when a fresh peptide solution is used, they also produce a similar outcome. Each of them exerts a weakness: for the Cys-peptides it is the disulfide formation and the consequent need of TCEP, for the Aox-peptides it is the presence of an unknown byproduct in solution. Taking into account that these ligations are studied to produce a functionalized textile, the optimization of the process from an industrial point of view should also be considered. The use of a fresh solution of a Cys-peptide allows to conjugate a peptide to an oxidized piece of cotton optimally, and without the presence of unknown species in the reaction mixture. On the other hand, **A1** does not show any degradation

of the peptide solution and does not require the use of additional molecules. Both these characteristics would be much appreciated in an industrial context due to the consequent higher suitability for a re-use of **A1**.

A2 and the double check of peptide loading

The Kaiser test allows to check if the peptide is effectively bound to cotton but does not quantify the amount of peptide. At the same time, **C1** and **A1** allow the evaluation of the peptide loading during the synthesis, but they do not permit to the check if , after the washing and drying of the cotton, the amount of peptide covalently bound to the cotton remains the same. Thus, **A2** was designed:

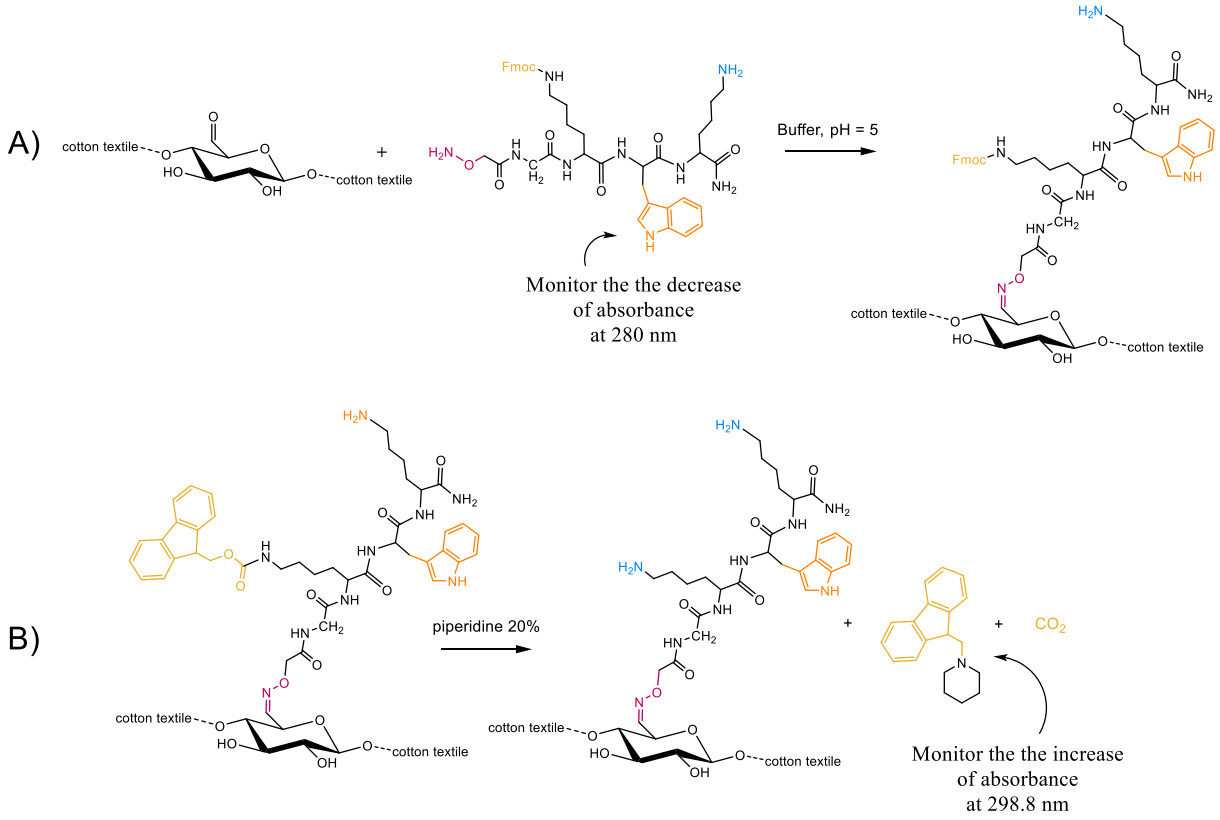


*Fig. 57: (A) Oxime bond formation with **A2**. (**B**) Release of Fmoc and CO_2 in solution upon piperidine treatment.*

Similarly to **A1**, **A2** has an Aox as the conjugating group and in addition to the Trp side chain, it also has a Fmoc group covalently bound to a Lys. In this way the Trp would allow the calculus of the peptide loading thanks to the decrease of its absorbance upon addition of the oxidized cotton. On the other hand, the removal of the Fmoc group from the peptide would allow the calculation of the peptide loading on the functionalized, washed and dried cotton.

Even though the freeze drying performed after the synthesis of **A2** determined the almost complete conversion to the +40 byproduct, all the obtained solid was dissolved in an acidic buffer to perform the conjugation with

cotton. In order to maximize the peptide-cotton ratio, the chosen weight of the cotton was the minimum possible to be handled and weighted (5 mg).

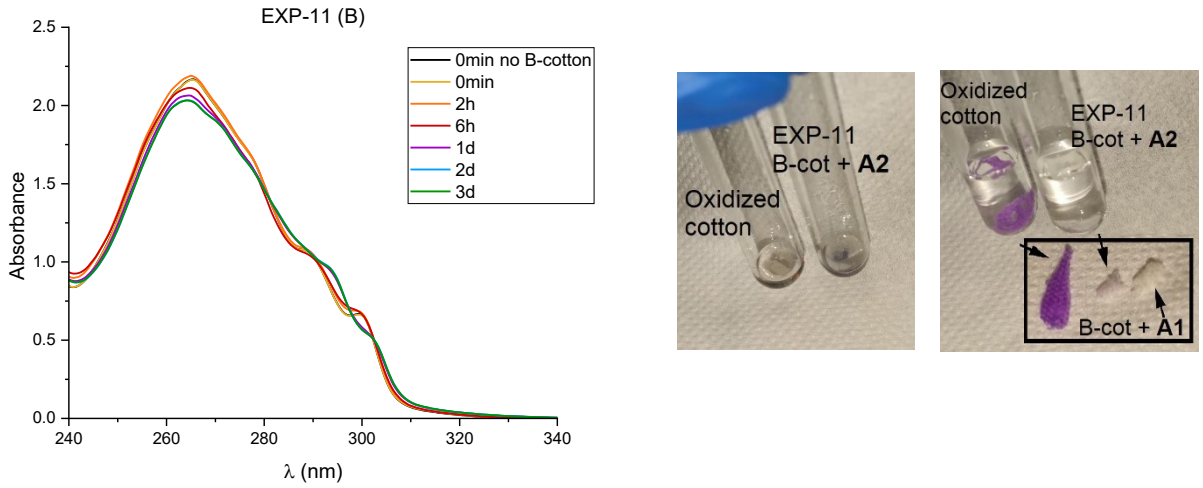


*Fig. 58: **On the left**: UV-vis spectra of the EXP-11 reaction. **In the center**: Kaiser test; **On the right**: Schiff test. Both tests were done after the removal of the Fmoc group on the functionalized cotton.*

Unfortunately, the spectrum of Fmoc covers the one of Trp (Fig. 58). Thus, the peptide loading obtained with the decrease of the absorbance at 280 nm could not be calculated. But still a decrease in the general absorbance was detected. After two days the reaction was complete. This was deduced by the stop in the decrease of absorbance for the peak at 260 nm. Therefore, after three days the functionalized cotton was removed from the solution and washed with water and acetone. After the cotton was dried, it was treated with a 20% piperidine solution in DMF for two times; the cotton is shaken 5 and 15 minutes, respectively. The piperidine solution was collected in a 10 mL graduated flask and diluted with DMF. The absorbance at 289.8 nm is 0.251 and thus the peptide loading is 0.0794 mmol/g. This demonstrates that **A2** was linked to the cotton. The value is slightly lower than the ones obtained from the measurements with **C1** and **A1**. After the Fmoc removal the Kaiser and Schiff test were performed: the first resulted blue, whereas the other resulted slightly pink. Therefore, the low value of the peptide loading could be due to a low peptide-cotton ratio, to the higher steric hindrance of the peptide or to an effective peptide loss during the washing. The last hypothesis could not be excluded due to the inability to monitor the reaction at 280 nm as previously done with **C1** and **A1**.

Overall, the results obtained with this peptide are coherent with the previously obtained with **C1** and **A1.** But the difficulties in its synthesis and the inherit impossibility to monitor the reaction at 280 nm makes the design of this peptide quite ineffective.

Conjugation of antibacterial peptides

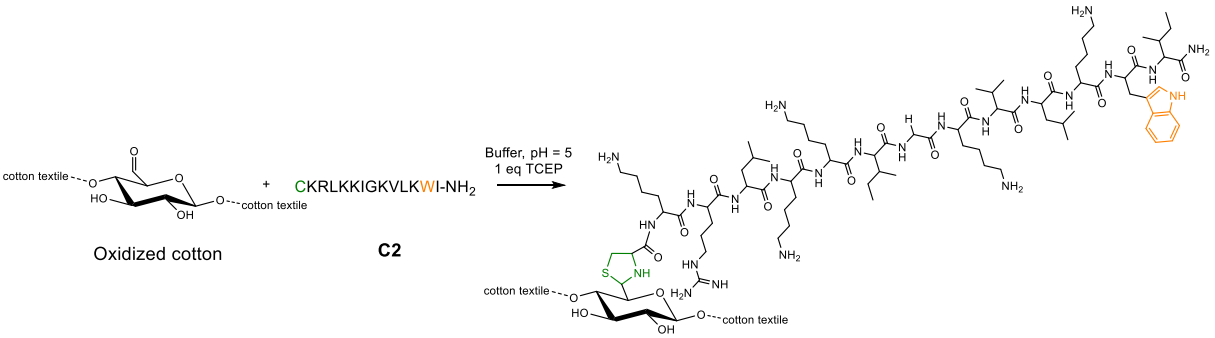


Fig. 59: Functionalization of the oxidized cotton with Cys-PMAP-36(12-24) trough thiazolidine bond formation.

The solid obtained after the purification of **C2** was not much (28 mg). Moreover, the successive biologic analysis required the cotton dimensions to be fixed (a circle with a diameter either of 1.5 cm or 1 cm), thus the mass of the cotton could not be below 16 mg. But still the some peptide-cotton conjugations could be performed.

All the peptide was dissolved in the acid buffer (pH =5) in a concentration of 1.46 mM. Then 1 eq TCEP and A-cotton (1.5 cm diameter) were added (EXP-12). The same solution was used also to conjugate a piece of B-cotton (1 cm diameter). Similarly to what was done with **C1**, one additional equivalent of TCEP was added before the beginning of the reaction (EXP-13). In both cases the peptide-cotton ratio was only around 0.5 mmol/g. The presence of a Trp permitted to monitor the reaction and a decrease in the absorbance at 280 nm could be effectively detected. Both reactions stopped after one day. This was a confirmation of the previous results with **C1**: a peptide-cotton ratio of 0.5 mmol/g is sufficient for the reaction to take place and a concentration of 1-2 mM determines lower reaction times. The loading of EXP-12 and EXP-13 were similar to each other and both much lower than the ones obtained with **C1**. Probably this is due to the higher steric hindrance of **C2**.

Sample	Peptide-cotton ratio (mmol/g)	Concentration (mM)	Loading (mmol/g)	Time
EXP-12 (A)	0.525	1.46	0.0386	1 day
EXP-13 (B)	0.570	1.38	0.0416	
EXP-14 (B)	0.328	2.35	0.0187	

Table 13: Summary table for EXP-12, -13 and -14.

To perform one experiment more, after EXP-13 the solution was freeze-dried and the obtained solid was re-dissolved in the buffer in a concentration of 2.35 mM. After one equivalent of TCEP was added to the solution, a piece of B-cotton (1.5 diameter) was introduced into the solution. In this case the obtained peptide-cotton

ratio was 0.328 mmol/g. Interestingly also in these conditions the reaction took place and a peptide loading of 0.0187 mmol/g was obtained. This further confirms what was observed with **C1**: a higher concentration determines a lower reaction time but does not affect the peptide loading, whereas a lower peptide-cotton ratio could determine a lower peptide loading.

Neither the Kaiser or Schiff test was performed on these cottons because all the obtained functionalized cotton samples were sent to the University of Trieste to perform the biologic tests.

3.7 ANALYSIS OF THE FUNCTIONALIZED COTTON

Once the functionalized cottons were obtained, some tests were performed: selected cotton pieces were analyzed by FT-IR spectrometry to further confirm the presence of the peptides on cotton; the resistance of the thiazolidine and oxime bond in different media was tested and biologic analysis were performed on the pieces of cotton functionalized with **C2**.

FT-IR spectrometry

Besides cotton and oxidized cotton (A and B), the functionalized cotton from EXP-3, -6 and the 2nd re-use of EXP-7 were subjected to FT-IR spectrometry. The last two were chosen because they had the highest peptide-loading for their type of cotton, whereas the cotton from EXP-3 was selected to investigate if a lower peptide loading could determine a difference in the spectrum. All the samples were finely chopped and incorporated into KBr pellets to perform the measure.

The most relevant modes of vibration of the amide bond are listed in the following table[94]:

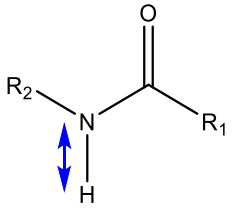
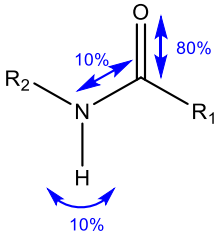
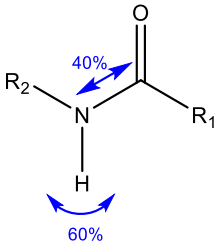
		Type of vibration	**Wavenumber (cm^{-1})**
Amide A		NH stretching	~ 3300
Amide I		C=O stretching	1600-1690
Amide II		CN stretching, NH bending	1480-1575

Table 14: Principal modes of vibration of the amide bond. The percentage shows how the single vibration contributes to the vibrational mode[94]

As shown in Fig. 60, the FT-IR spectra of cotton is coherent with the data found in the literature.[95] In particular the absorption in the region 3200-3600 cm^{-1} and 1000-1200 cm^{-1} confirms the presence of the hydroxyl

moieties- In fact, the first corresponds to the O-H stretching, whereas the second corresponds to the C-O stretching or the O-H deformation. Aliphatic C-H are also present in the cotton structure and their stretching is visible around 2800 cm^{-1}. The band at 1600 nm is attributable to the bending of the H_2O that could deposit on the cotton surface. Furthermore, no difference could be detected between the spectra of A- and B-cotton.

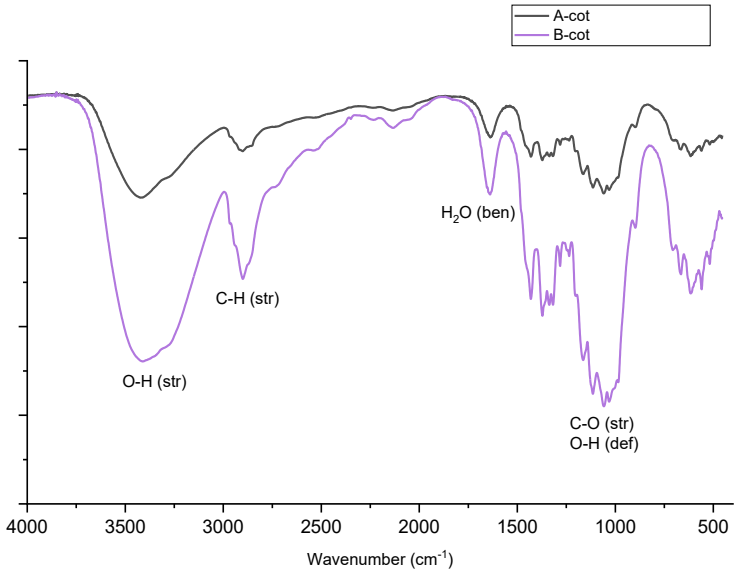


Fig. 60: FT-IR spectra of A and B-cotton

Unfortunately, the presence of the aldehydes on the oxidized cottons could not be detected. In fact, they are characterized by a C=O stretching in the range 1750–2150 cm^{-1}, a C-H stretching in the range 2700–2800 cm^{-1} and a C-H deformation in the range of 110-1300 cm^{-1}.[95] But they are all covered by the bands of cotton and water. On the other hand, the only mode of vibration of the peptide bond that is clearly visible in all the tested samples is the *Amide II* at around 1500 cm^{-1} (Fig. 61). The *Amide A* and *Amide I* are covered by the O-H stretching and H_2O bending, respectively.

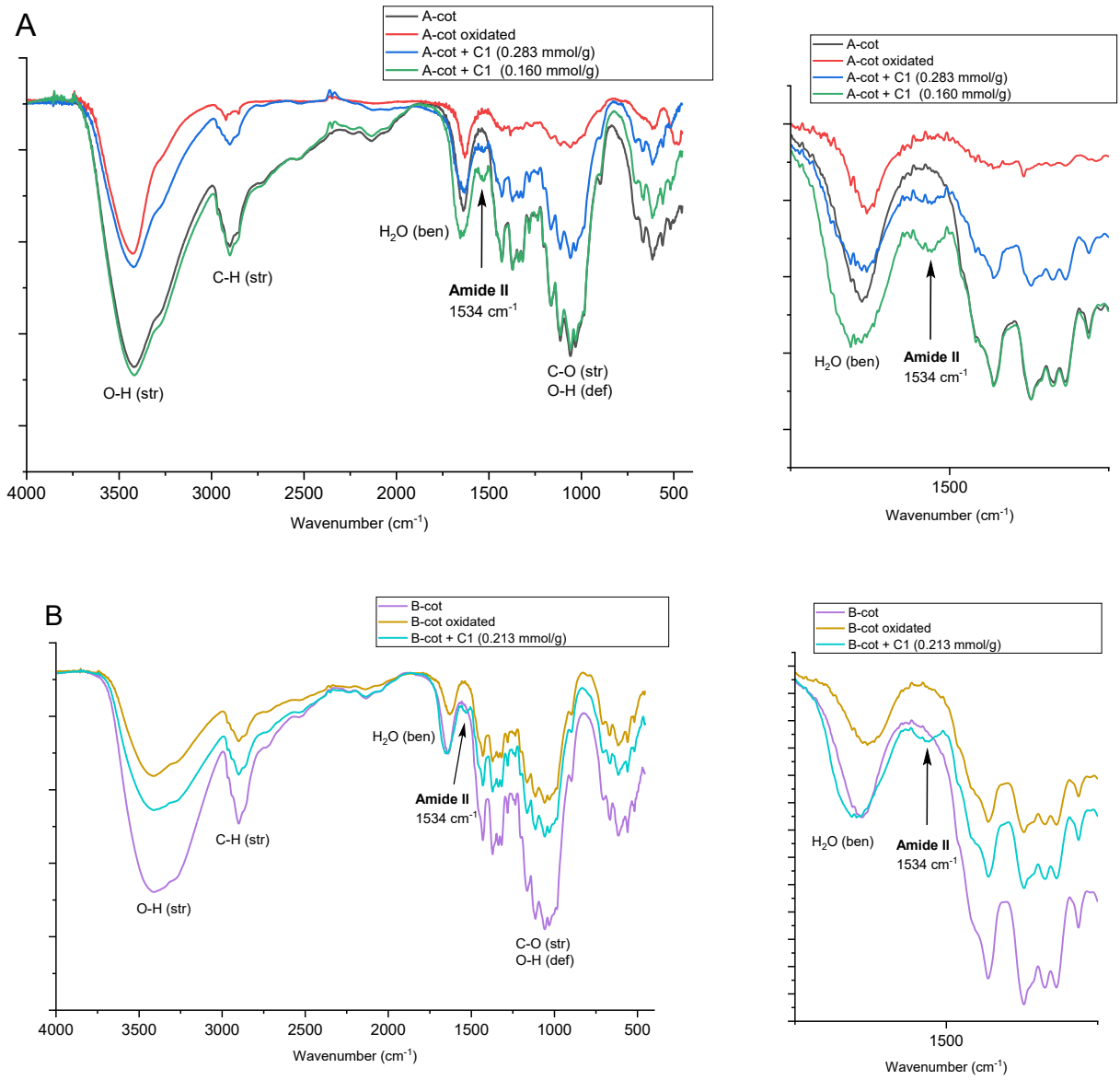


*Fig. 61: FT-IR spectra of (**A**) A-cotton (black); oxidized A-cotton (red); A-cot + C1 from EXP-3 (green) and EXP-6 (blue). (**B**) B-cotton (purple); oxidized B-cotton (yellow) ; B-cot + C1 from EXP-7 – 2nd re-use (light blue)*

In conclusion, the peptide is confirmed to be bound on cotton and no differences could be detected between A-cotton and B-cotton. Also, the difference in the amount of peptide bound in EXP-63 and EXP-6 was not appreciable with this method of investigation.

Resistance of the conjugation

The stability of the thiazolidine and oxime bonds on B-cotton were tested at different pH and with EtOH. To do so, pieces of cotton from EXP-4 (**C1**) and EXP-5 (**A1**) were added to the chosen solution, to test the thiazolidine and oxime bonds respectively, and the absorbance at 280 nm was monitored for 3 days. In particular:

- A citrate buffer at a pH of 3 and a 3M HCl solution were used to test the resistance of the ligations at **acidic pH**.
- The solvent used to perform the biologic analysis is a PBS buffer at pH 7. Thus, this solvent was used to test the ligation at **neutral pH**.
- A carbonate buffer at a pH of 10 was used to test the resistance of the ligation at **basic pH.**
- A **70% EtOH** solution is used for the sterilization of the cotton samples before the biologic analysis. Thus, this solution was used to check whether this treatment could affect the functionalization of the textile.

In all the tested solvents some peptide release in solution could be observed as an increase of the Trp absorbance at 280 nm. As shown in the examples of Fig. 62 in some media (as PBS) the release in solution was negligible, whereas in others (like 3M HCl) the release was rather significant.

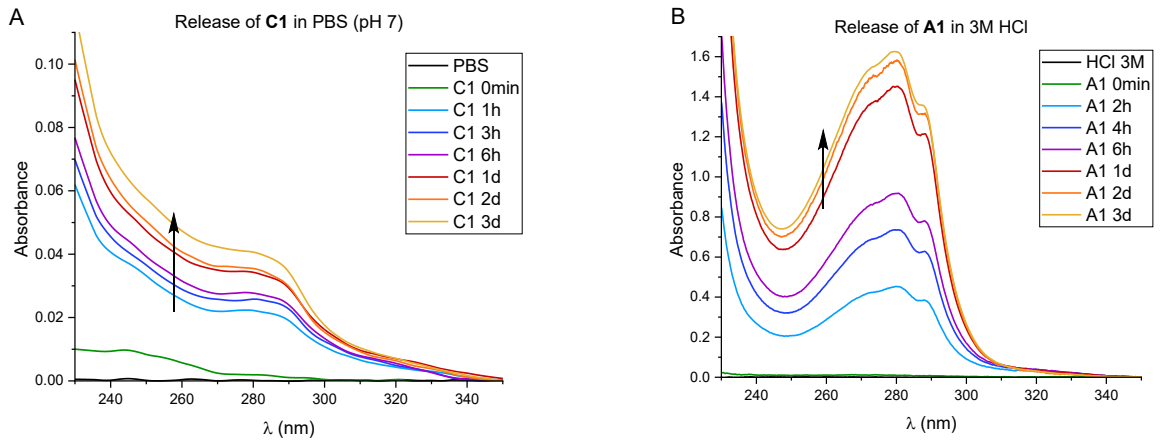


*Fig. 62: UV-vis spectra (**A**) of **A1** in PBS (**B**) of **A1** in HCl*

To quantify the amount of peptide released in solution, the percentage of peptide loss was used:

- The Lambert-Beer law permits to calculate the concentration of Trp (and thus of the peptide) in solution as $C = \frac{A}{\varepsilon \cdot l}$; where C is the concentration, A the absorbance, ε the extinction coefficient and l the optical path. The lower and upper thresholds for the validity of the law were set at 0.05 and 1 in absorbance, respectively.
- $n_{released-peptide} = C \cdot V$; where V is the volume of the solvent added to the functionalized cotton.
- The weight of the cotton and the peptide loading are known from the conjugation reaction of EXP-4 and EXP-5. For **C1**-cotton 0.100 mmol/g is and for **A1**-cottoin is 0.126 mmol/g. Thus, the amount of peptide bound to cotton is also known.
- $\%\, peptide\ loss = \frac{nreleased-peptide}{nbound\ peptide} \cdot 100.$

The results obtained are summarized in Fig. 63 and table 15:

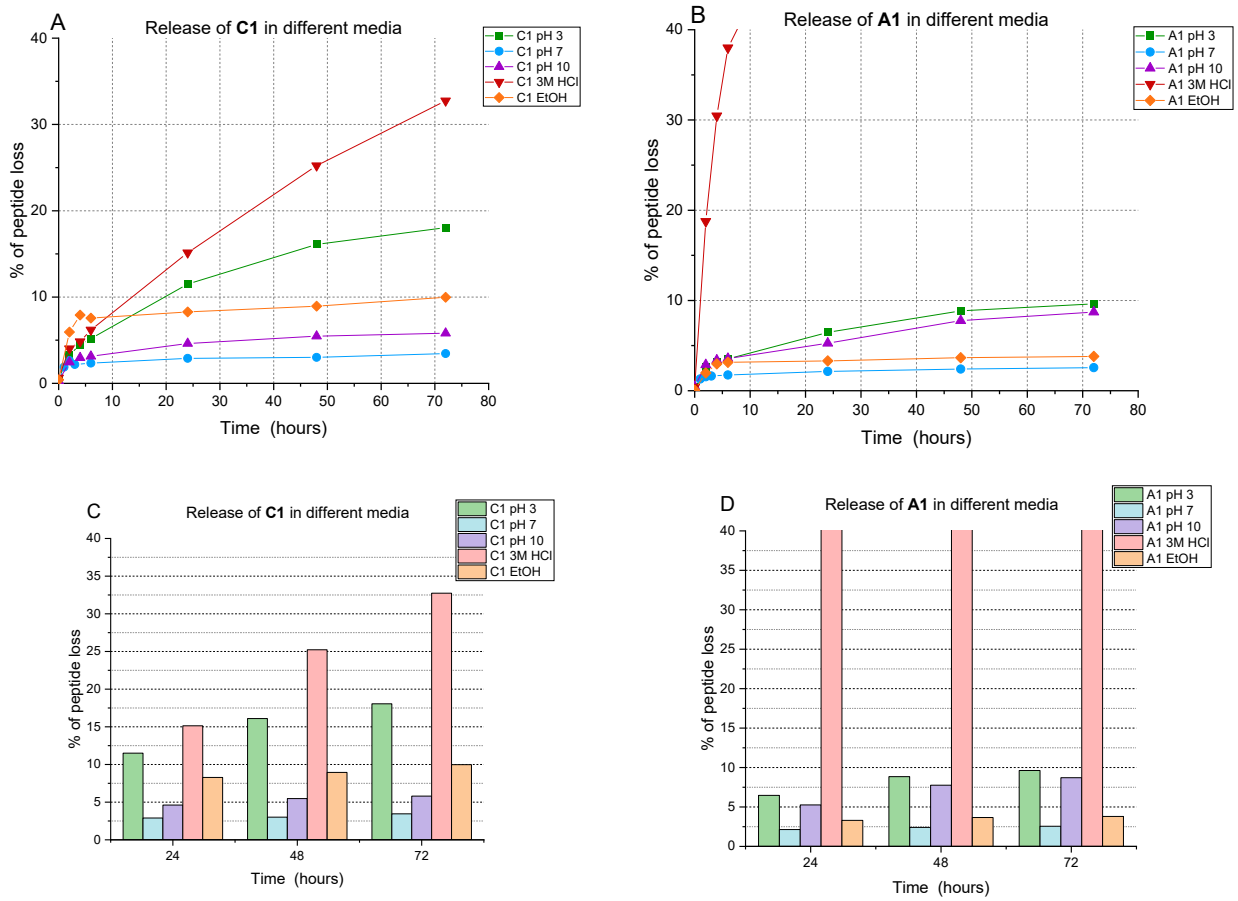


*Fig. 63: Percentage of peptide loss over time. **(A)** for **C1**; **(B)** for **A1.** Percentages in details at 24, 48 and 72 hours for **(C) C1** and **(D) A1**. The graph does not show data over 40% of peptide loss due to the chosen upper threshold in absorbance. In fact, with absorbances higher than 1 the calculation of the concentration in solution, and consequently also the calculation of the peptide loss, are not accurate anymore.*

	Loss of peptide after 3 days	
	C1	**A1**
pH 3	18%	10%
pH 7	<4%	<3%
pH 10	6%	9%
HCl	33%	> 40%
70% EtOH	10%	4%

Table 15: Percentages of peptide loss after 3 days in different media.

The higher peptide loss was obtained in 3M HCl both for **C1** and **A1**. But after 3 days the peptide loss of **C1** was of 33%, whereas the one of **A1** was much higher (Fig. 63). In PBS the loss of peptide is negligible in both cases, thus in the conditions used for the biologic analysis both types of bond can be considered highly stable. At basic pH the oxime bond seemed slightly less stable than the thiazolidine. At acidic pH the opposite happened: the thiazolidine bond was slightly less stable than oxime. This is coherent with the reported ranges of the thiazolidine and oxime bond which are of 5 to 8 pH and 3 to 7 pH, respectively (see paragraph 2.2) The behavior in EtOH is similar to that in the acidic conditions. But still the peptide loss was not higher than 10%. Considering that the sterilization procedure lasts for 1 hour and that **C1** and **A1** loss was around 6% and 2%, respectively, it can be performed without a significant loss of peptide.

It is also important to notice that a previous test done with B-cotton functionalized with **C1** (EXP-3) and a 70% EtOH gave slightly different results than the ones here reported. In this case the release was monitored only for 5 hours, and the obtained peptide loss was of 2%. This value is lower than the 8% peptide loss detected after 4 hours for EXP-4 cotton in 70% EtOH. The only difference between the two experiments was the temperature in the laboratory, which was higher for the second experiment. Thus, for the future also the role of temperature in the release of peptide should be tested.

Biologic analysis

The cottons functionalized with **C2** were sent to the University of Trieste to perform analysis in the bacterial biofilm. In a previous analysis, the antimicrobial activity of the peptide **C2**, Cys-PMAP-36(12-24), chosen for this work was evaluated against different bacterial strains (Tab. 15):

MIC (μM) for Cys-PMAP-36(12-24)

E.coli	S.aureus	A.Baumanni	K.pneumoniae	P.aeruginosa	S.Epidermidis
8	32	2	8	4	4

*Table 16: MIC (minimum inhibitory concentration) of **C2** with different strains of bacteria. As highlighted by the color change, the lowest MIC is obtained in the case of A.Baumanni and the highest with S.Aureus.*

The tests appointed *A.Baumanni* to be the most susceptible (of the tested bacterial strains) to **C2**, thus this bacterium was chosen to perform the tests with cotton.

The functionalized cottons stayed in the culture medium for 24 hours. Then, to evaluate the bacterial growth measurements of optical density were performed. The higher the optical density is, the higher is the bacterial growth.

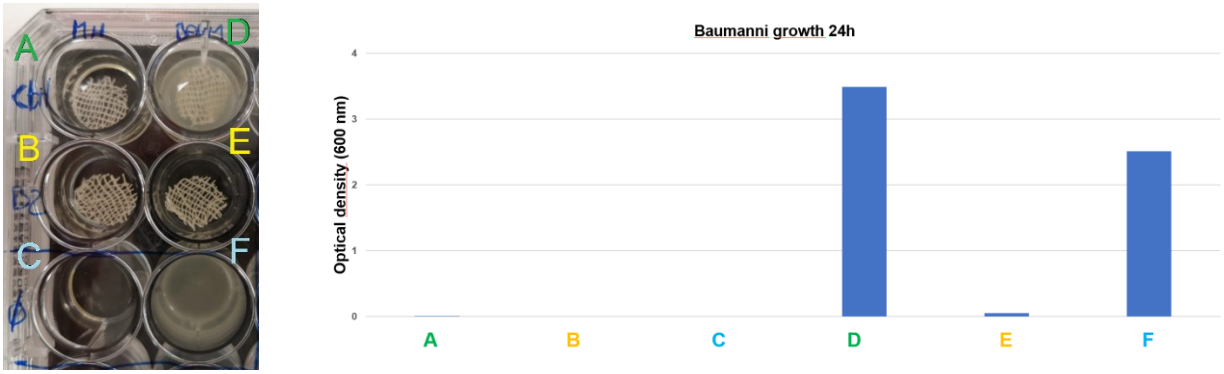


*Fig. 64: Biofilm growth on A-cotton. (**A**),(**B**) and (**C**) are the controls without the bacteria. (**D**), (**E**) and (**F**) are in the presence of A.Baumanni. Green labels indicate A-cotton. Yellow labels indicate A-cotton functionalized with **C2** (from EXP-12). Blue labels for the samples without any cotton.*

As shown in Fig. 64, *A.Baumanni* grew only in the absence of cotton or in the presence of the pure A-cotton. Whereas, in the presence of **C2**-cotton the bacterial growth was virtually null. This demonstrates the efficacy of cotton functionalized with **C2** as an antibacterial fabric. The Resazurine assay further confirmed it. In fact, in this assay the solution used for the previous test were subjected to a treatment that establishes the vitality of the bacteria in solution. If the solution remains blue the bacterium is not vital, whereas if it turns pink the bacteria is alive. This is quantified by fluorescence measurements.

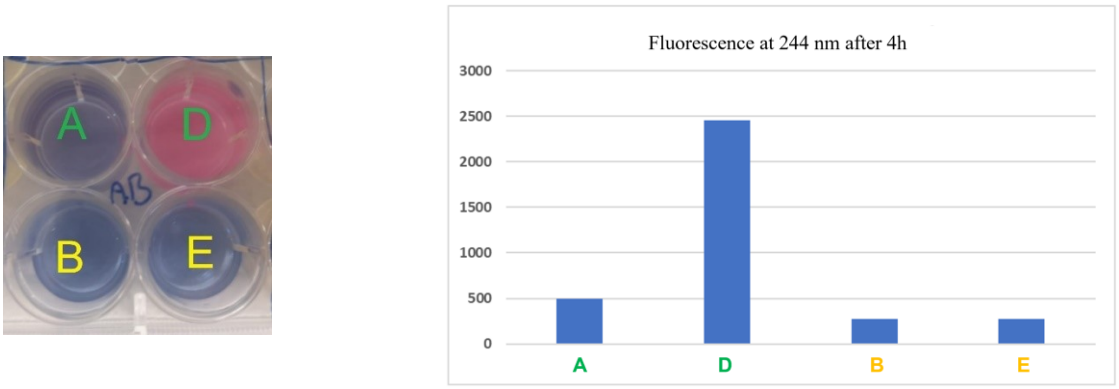


*Fig. 65: Resazurine assay on the solutions used for the biofilm assay. (**A**) and (**B**)are the controls without the bacteria. (**D**) and (**E**)are in the presence of A.Baumanni. Green labels indicate the solutions that contained A-cotton. Yellow labels indicate the solutions that contained A-cotton functionalized with **C2** (from EXP-12).*

As shown in Fig. 65, the solution where **C2**-cotton was present remained blue. Thus, the bacteria are not vital due to the presence of the antibacterial cotton. Whereas bacteria in the solution that contained the pure A-cotton were proved to be vital.

The same results were obtained with **C2**-cotton from EXP-13 and EXP-14. This also indicates that the peptide loading of 0.0187 mmol/g obtained with B-cotton in EXP-14 is sufficient to exert the antimicrobial properties of **C2**. Interestingly to notice the bacterial growth and vitality is slightly higher in the presence of pure cotton than in the absence of it, proving tissues to be potential centers of bacterial growth.

4. CONCLUSIONS

The growth of bacteria on textiles and the consequent transmission of them sparks the need of development of antimicrobial textiles. Both for environmental and efficienty reasons the durability of the so formed material should be maximized. Thus, in this context the use of covalent bonds between the tissue and the antimicrobial agent is an effective solution. For the same environmental reasons and its unique set of properties, the better choice of material is cotton, whereas due to the broad-spectrum mechanism of action and biocompatibility, AMPs are a more secure and environmental-friendly choice than common antibiotics.
In this thesis the thiazolidine and oxime bonds were investigated in the context of a peptide-cotton conjugation for the development of antimicrobial textiles. To do so, aldehydes moieties needed to be introduced on cotton through oxidation and the peptide chain N-terminal needs to be modified with a Cys or an Aox, respectively.

The enzymatic oxidation of cotton by the TEMPO/laccase/O_2 system was successful. In fact, it made possible to introduce an aldehyde at the C_6 of the glucose unit of cotton cellulose, at room temperature and in a slightly acidic aqueous solvent. The re-use of the solution was tested to evaluate if the environmental impact of this step could be diminished, and it was successful only at a certain extent. In fact, the degree of oxidation was decreased at each re-use. Given that this is probably due to the degradation of laccase, maybe some adjustment to the procedure could be investigated to maximize the oxidation potential of the solution. In particular lower times of reaction should be tested and, considering that the presence of an alcohol moiety slows the degradation of laccase[31], the time between two consecutive oxidations should be minimized.

Two types of peptides were used in this synthesis: “probe” and antimicrobic. Both were synthetized by SPPS. To allow the conjugation with the oxidized cotton, the first amino acid of the sequence is either Cys or Aox. The Cys ending peptides did not exert problems during the synthesis, whereas all the synthetized Aox ending peptides seemed to produce an unknown byproduct with forty m/z units more than the target peptide when placed in aqueous solvents. Thus, a further investigation on the nature of this byproducts is needed.

The peptide-cotton chemoselective conjugation was investigated especially with the probe peptides **C1** and **A1**. Both the thiazolidine and oxime formation takes place in an aqueous solvent at a slightly acidic pH, but the first also needs the presence of a reducing agent in solution to avoid the dimer formation. Following the reaction through the decrease of the Trp absorbance proved to be successful and permitted the calculus of the peptide loading on cotton. The effective success of the conjugation was confirmed both by colorimetric measurement and FT-IR spectra. The experiments with **C1** proved that there is not a particular difference in the peptide loading, and thus aldehyde content, between the three tested types of cotton and it is around 0.15 $mmol_{peptide}/g_{cotton}$; but confirmed that the re-use of the oxidation solution determines a lower aldehyde content. Furthermore, the fastening of the reaction by an increase in the peptide concentration was proved. This was

confirmed by the experiments with **A1**. For the same reasons described above, the possibility to re-use the peptide solution was investigated both with **C1** and **A1**. The results in this case were different. The **C1** solution seemed to degrade over time, maybe due to the presence of TCEP in a high excess, whereas the **A1** solution did not present this problem. These two peptides were also used to test the resistance of the thiazolidine and oxime bond in different media and the results were similar. The release at neutral pH is insignificant for both peptides, the thiazolidine bond is slightly more stable than oxime at a basic pH, whereas the oxime bond is slightly more stable at pH 3 and this is consistent with the ranges of stability found in the literature.[55] The EtOH tests proved that with either conjugation the sterilization process can process without significant loss in the peptide loading, although the lowest loss takes place in the case of the oxime bond.

The design of **A2** proved to be unsuccessful for its purpose of a double check on the peptide loading, but confirmed the results obtained with the reaction of **A1** and vanillin. The +40 byproducts do not interfere with the conjugation reaction, even if they are present at high excess. Instead of following the absorbance of two different species, for the future it could be interesting to develop a probe peptide with Trp as the UV-active molecule and a fluorescent probe group. In this way, the results obtained with two different groups would not interfere one with another.

The functionalization of cotton with **C2** was successful, even at low peptide-cotton ratios. This confirms the efficacy of the thiazolidine ligation. The **A3** conjugation is postponed until the nature of the +40 byproduct is revealed. The biologic analysis also proved the **C2**-cotton material to be effective against *A.Baumanni*. Considering that there is almost no available drug based therapy for the treatment of this bacterium[96], the obtained results are rather significant. Tests for the antimicrobial effectiveness over repetitive use are under investigation.

Overall, taken apart the synthesis of the peptides, the processes of conjugation between Cys and Aox ending peptides to cotton take place at mild conditions. The main drawback of the thiazolidine bond formation is the formation of dimers and the consequent presence of TCEP in solution, whereas the only drawback of the oxime formation in this context is the presence of an unknown byproduct. Taken into account the absence of degradation of the **A1** solution with respect to the **C1** solution, the no need of additives and the slightly lower release in EtOH, oxime is probably a better alternative than thiazolidine for the production of an antimicrobial cotton textile.

5. EXPERIMENTAL PART

5.1 MATERIALS AND INSTRUMENTS

Reagents and solvents

Brand	Reagents and solvents
Aldrich	TEMPO, Fmoc-Gly-OH, Fmoc-Trp(Boc)-OH, TCEP
Carlo Erba	Methanol, Acetonitrile, NaH_2PO_4, KH_2PO_4, Sodium citrate, $NaHCO_3$
Eurisotop	DMSO d6
GL Biochem	Fmoc-OSu, Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-Ile-OH, Fmoc-Val-OH
Honeywell	Piperidine
Iris biotech	Fmoc Rink Amide AM, Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH, HOBt, HBTU, Oxime, DIC
Riedel-den Haen	Sodium acetate, Citric Acid
Sigma	Schiff reagent, Laccase from "Trametes Versicolor", DIPEA
Sigma-Aldrich	Kaiser test kit, Acetone, Acetic acid, Diethylether, Hydrochloric acid, Ethanol, DMF, TFA, Boc-Aminooxiacetic acid-OH, Fmoc-Lys(Dde)-OH, NaCl, KCl, Na_2CO_3

Cotton	Source
A	Medical gauze from Santex Spa
B	Organic cotton from Piave Maitex Srl; $g/m^2 = 120$
C	Raw cotton from Santex Spa

Instruments

High performance liquid chromatography (HPLC)

A "VWR HITACHI Cromaster" was used for HPLC analyses. It is composed of the following modules: VRW HITACHI UV-Detector 5410 (simultaneous 280 nm and 216 nm wavelenghts), Auto sampler 5260 and Pump 5410. An C_{18} reversed phase Phenomenex 100 Å, 5µ, 250 x 4.60 mm column that has a 1mL/min flow was used.

Eluents: A 90% H_2O / 10% ACN + 0.05% TFA

B 10% H_2O / 90% ACN + 0.05% TFA

LC-MS

An "Agilent technologies 1260 Infiniti II" was used for HPLC-MS analyses. It is equipped with a "Poroshell 120 EC-C18 2,7 µm, 100x4,6 mm C_{18} reversed phase column.

Eluents: A 100% H_2O + 0.1% TFA

B 100% ACN + 0.1% TFA

The chromatographic module is connected with a "Agilent technologies quadrupole LC-MS 6130" mass spectrometer that uses ESI ionization.

Flash chromatography

A medium pressure flash chromatograph (Biotage – Isolera Prime) was used to purify some of the peptides. The sample was absorbed to a pre-column before the elution. A "C_{18} SNAP 12g" column with a maximum volume of 15 mL and a 12 mL/min flow was used. A specific gradient of elution can be selected, and the eluted sample is automatically collected in separated tests tubes. The instrument is equipped with a UV-detector that continuously monitors at 216 nm and 280 nm the outgoing sample.

Eluents: A 100% H_2O + 0.05% TFA

B 10% H_2O / 90% CAN + 0.05% TFA

At the end, the fractions of interest were freeze-dried (after the removal of ACN).

UV-vis absorption spectrophotometry

A "Shimadzu UV-vis 250 1PC" interfaced with a PC was used to collect the UV-vis spectra. A 350-230 nm interval, 2nm slit and a 0.1nm sampling interval were used. Depending on the experiment quartz "Hellma Analytics" cuvettes with an optical path of 1cm or 1mm were used.

NMR

A "Bruker Advance DRX 400 MHz" was used to collect the H^1-NMR spectra. The chemical shifts (d) are expressed in parts per million (ppm) with respect to the reference signal of the TMS and the coupling constants are expressed in Hertz.

FT-IR spectrometry

A "Nicolet nexus 670" spectrophotometer was used to collect the FT-IR spectra. The cotton samples were mechanically frayed and incorporated in a KBr pellet. The chamber were the samples sit are maintained under a constant N_2 flow to minimize the contributions of H_2O and CO_2. The spectra were collected through 20 scans in the range 4000-400 cm^{-1}, with a resolution of 2 cm^{-1}. The spectra were collected by Dr. Renato Schiesari from the Chemical Science Department of the University of Padova.

Automatic synthesizer

A "Biotage – Syro Wave" synthesizer was used to synthetize some of the peptides. The resin and the amino acids were weighted manually. All other operations are carried out automatically by the instrument.

5.2 KAISER AND SCHIFF TESTS

Kaiser test

Cotton: A dried cotton thread of ca. 0.5cm is cut and put into a test tube. 20 mL of each solution are added (potassium cyanide in pyridine, 80% phenol in ethanol, and 6% ninhydrin in ethanol). The solution is heated with a heat gun to about 100°C for approx. 10 seconds. After 5 minutes the color of the cotton is checked.
Resin: A dozen grains of resin are dried with DCM and put into a test tube. 20 mL of each solution are added (potassium cyanide in pyridine, 80% phenol in ethanol, and 6% ninhydrin in ethanol). The solution is heated with a heat gun to about 100°C for approx. 10 seconds. After 5 minutes, the color of the grains is checked.

Schiff test

A cotton piece is cut (about 1cm long) and soaked with approximately 1mL of Schiff reagent is added. After 10 minutes the colour of the cotton is checked.

5.3 PRE-TREATMENT OF THE COTTON

Mercerization of cotton

The cotton is dipped in a 1% NaOH solution. The solution is heated to reflux. The cotton is left for some time in the solution. When the cotton is rich in paraffines the solution becomes yellow. The solution is changed over time and the treatment continues until the NaOH solution does not change color anymore. Then, the cotton is washed with water until the pH turns back to neutral. At the end the cotton is washed with methanol to ease the drying process.
This process was done for **A**- and **C**-cottons by Dr. Stefano Scapin.[6]

Oxidation of cotton

1st oxidation: 20 U/g_{cotton} of Laccase and 8% (m/m_{cotton}) of TEMPO were dissolved in $30mL/g_{cotton}$ of NaOAc/AcOH buffer (50mM, pH = 5). Then the cotton was added to the solution. After 2 days of stirring at room temperature, the cotton was removed from the solution, washed with 20mL x 3 of NaOAc/AcOH buffer (the washing solution is put back in the flask), 20mL x 5 of water and 20mL x 5 of acetone. The cotton was dried in the desiccator for 1h and stored in a plastic vial. This was done for A-, B- and C-cotton
2nd and 3rd oxidations: The cotton was added to the Laccase/TEMPO solution of the previous oxidation and stirred at room temperature for 4 days. Then it was removed and washed with 20mL x 3 of NaOAc/AcOH buffer (the washing solution is put back in the flask), 20mL x 5 of water and 20mL x 5 of acetone. The cotton was dried in the desiccator for 1h and stored in a plastic vial. The 2nd oxidation was done for all the cottons, the 3rd one was done only for A-cotton

5.4 SOLID PHASE PEPTIDE SYNTHESIS

Depending on the experiment, the solid phase peptide synthesis was performed manually, semi-automatically or with an automatic synthesizer. In either case, the basic principle is that the amino acidic chains grow onto the resin one amino acid by one, from the C-terminal amino acid to the N-terminal one.[83] Furthermore, for each method the synthesis can be rationalized in steps that are repeated.

The **manual synthesis** is done in a syringe with a polyethylene filter at the smallest end and one cap at each end. It can be summarized with the following steps:

1) SWELLING: the resin is submerged in DMF and is shaken for at least 30 minutes. This allows the reagents to better penetrate into the grains of the resin.
2) FMOC REMOVAL: the resin is treated with a 20% v/v piperidine/DMF solution and shaken for 5 minutes. The procedure is repeated a second time but shaking for 15 minutes. Then the resin is washed 6 times with DMF. This procedure is performed on Fmoc-protected resins and after each AA-coupling.
3) DDE REMOVAL (when needed): the resin is treated with a 2% v/v hydrazine monohydrate/DMF solution and shaken for 3 minutes. This procedure is repeated 4 times. Then the resin is washed 6 times with DMF to completely remove the solution.
4) AA COUPLING: all the peptides are synthetized through the Fmoc/Boc strategy. Therefore, if not differently noted, all the used amino acids are Fmoc-protected. To proceed with the coupling 3eq, with respect to the mmoles of resin, of amino acid and of Oxime (3eq) are dissolved with the minimum amount of degassed DMF. Then 3eq of DIC are added and the solution. This is done to activate the AA. After 5 minutes of stirring, the activated AA solution is added to the resin and shaken for 1 hour. At the end, the resin is washed 6 times with DMF to remove the excess of reagents.
5) FMOC COUPLING (when needed): 3eq, with respect to the mmoles of resin, of Fmoc-OSu are dissolved in the minimum amount of DMF. Then, 6eq of DIPEA are added. The solution is added to the resin and shaken for 1h. At the end, the resin is washed 6 times with DMF to remove the excess of Fmoc-OSu.
6) DCM DRYING: after the peptide is synthetized, the resin is washed 6 times with DCM and put in the desiccator.
7) RELEASE FROM THE RESIN: it is the removal of the peptide from the resin and the contemporary deprotection of the side chains. The resin is shaken for 2 hours with a solution of TFA/DODT/H_2O/TIS in 94/2.5/2.5/1 ratio or TFA/TIS/H_2O in of 95/2.5/2.5 ratio, used in the presence of Cys or in its absence, respectively. Then, the solution is added to a vial containing Et_2O (in a volume at least 10 times the volume of the release solution) to precipitate the peptide. Subsequently, the solution in centrifuged for 10 minutes at 6000 rpm and the supernatant is removed. The obtained solid peptide is washed with Et_2O, centrifuged as described above and the supernatant removed; this last procedure is repeated for 3 times. Then the resin is put in the desiccator.

The removal from the resin can be done when the peptide chain is not completed to check the synthesis: a dozen of grains of resin are taken, if needed dried with DCM and the above-mentioned procedure is performed.

The **semi-automatic synthesizer** fills, shakes and empties the glass reactor automatically following a pre-determined program. The reactor is equipped with a porous septum that let the liquids though and keeps the resin in. Three of the six available filling lines were used: one for DMF, one for the 20% piperidine/DMF solution and one for DCM. The pre-determined functions of the instruments are:

- FILL: fills the reactor
- REACTION: shakes the reaction for a given time
- EMPTY: empties the reactor using a vacuum pump
- WASH: fills the reactor, shakes for 1 minute and empties the reactor

The programs that were used were:

1) SWELL: FILL 5mL DMF; REACTION for 20 minutes; EMPTY; FILL 5mL DMF; REACTION for 20 minutes; EMPTY
2) DEPR + WASH: FILL 5 mL PIP/DMF; REACTION for 5 minutes; EMPTY; FILL 5mL PIP/DMF; REACTION for 15 minutes; EMPTY; WASH x5 with 5mL DMF
3) COUP + WASH: REACTION for 1 hour; EMPTY; WASH x5 with 5mL DMF
   In this case the solution containing the amino acid and the activators was added manually
4) WASH DCM: FILL 5 ml DCM; REACTION for 4 minutes ; EMPTY; FILL 4 ml DCM; REACTION for 4 minutes ; EMPTY; WASH x4 with 5 ml DCM

The **automatic synthesizer** works similarly the semi-automatic one. The main differences are:

- It can directly withdraw the activated AA solution
- It adds the 20% piperidine solution (in DMF) and the HOBt/HBTU solution in DMF automatically to the dissolved AA in DMF

Synthesis of probe peptides

Peptide C1

C1: H-Cys[1]-Gly[2]-Trp[3]-Lys[4]-NH_2

"Fmoc-Rink Amide AM" with a theoretical loading of 0.65 mmol/g was used. For every step 3 eq of amino acid, Oxime and DIC were used, with respect to the moles of the resin.

	MW (g/mol)	Moles (mmol)	Mass (mg)		Mass of Oxime (mg)	Volume of DIC (mL)
Fmoc-Rink-Amide AM	-	0.325	500		-	-
Fmoc-Lys(Boc)-OH	468.5	0.975	456.8	+	138.6	151.0
Fmoc-Trp(Boc)-OH	526.6	0.975	513.4	+	138.6	151.0
Fmoc-Gly-OH	297.3	0.975	289.9	+	138.6	151.0
Fmoc-Cys(Trt)-OH	585.7	0.975	571.1	+	138.6	151.0
Total					554.4	604.0

The synthesis was done manually and following steps were done:

1. SWELLING
2. FMOC REMOVAL
3. Lys^4 – COUPLING
4. FMOC REMOVAL
5. Trp^3 – COUPLING
6. FMOC REMOVAL
7. Gly^2 – COUPLING
8. FMOC REMOVAL
9. Cys^1 – COUPLING
10. FMOC REMOVAL
11. DCM DRYING
12. RELEASE OF 9 mg
13. RELEASE of all the resin

The obtained solid was dissolved in 5mL of H_2O + 0.1% TFA and freeze dried.

Yield after freeze drying: 88%.

HPLC: solvent H_2O + 0.1% TFA. Gradient of 0-30% B in 30 minutes. t_R = 6.49 min

LC-MS: solvent H_2O + 0.1% TFA. Gradient of 0-30% B in 30 minutes. t_R = 5.69 min; $[M+H]^+$ = 492.3 m/z

^{1}H-NMR (400 MHz, DMSO d6): δ 10.81 (s, 1H, H3(N) Trp^3), 8.78 (d, 1H, NH Gly^2), 8.22 (m, 1H, NH Cys^1), 8.21 (m, 1H, NH Trp^3), 8.04 (t, 1H, NH Lys^4), 7.63(m, 2H, εCH$_2$(N) Lys^4), 7.60 (m, 1H, H7 Trp^3), 7.32 (m, 1H, H4 Trp^3), 7.16 (m, 1H, H2 Trp^3), 7.06 (m, H5 Trp^3), 6.98 (m, 1H, H6 Trp^3), 4.59 (m, 1H αCH Trp^3), 4.17 (m, 1H, αCH Lys^4), 4.01 (m, 1H, αCH Cys^1), 3.91 and 3.68 (m, 2H, αCH$_2$ Gly^2), 3.20 and 2.97 (m, 2H, βCH$_2$ Cys^1), 3.16 and 2.94 (m, 2H, βCH$_2$ Trp^3), 2.75 (m, 2H, εCH$_2$ Lys^4), 1.66 and 1.27 (m, 2H, γCH$_2$ Lys^4), 1.55 (m, 2H, βCH$_2$ Lys^4), 1.50 (m, 2H, δCH$_2$ Lys^4).

Peptide A1

A1: H-Aox[1]-Gly[2]-Trp[3]-Lys[4]-NH_2

"Fmoc-Rink Amide AM" with a theoretical loading of 0.65 mmol/g was used. For every step 3 eq of amino acid, Oxime and DIC were used, with respect to the moles of resin.

	MW (g/mol)	Moles (mmol)	Mass (mg)		Mass of Oxime (mg)	Volume of DIC (mL)
Fmoc-Rink-Amide AM	-	0.455	700		-	-
Fmoc-Lys(Boc)-OH	468.5	1.365	639.5	+	194.0	211.4
Fmoc-Trp(Boc)-OH	526.6	1.365	718.8	+	194.0	211.4
Fmoc-Gly-OH	297.3	0.684	202.9	+	97.0	105.7
Fmoc-Aox-OH	313.3	0.684	213.8	+	97.0	105.7
Total					582.0	634.2

The synthesis was done semi-automatically for the first two amino acids:

1. SWELL
2. DEPR + WASH
3. Lys^4 – COUP + WASH
4. DEPR + WASH
5. Trp^3 – COUP + WASH
6. DEPR + WASH
7. WASH DCM

Then the resin was split in two: the first 350 mg were used for this synthesis; the rest was used for the synthesis of **A2**. The rest of the synthesis was done manually and following steps were done:

1. SWELLING
2. Gly^2 – COUPLING
3. FMOC REMOVAL
4. Aox^1 – COUPLING
5. FMOC REMOVAL
6. DCM DRYING
7. RELEASE OF 11 mg
8. RELEASE of all the resin

Yield: 73%

HPLC: solvent MeOH. Gradient of 0-30% B in 30 minutes. t_R = 6.89 min

LC-MS: solvent MeOH. Gradient of 0-80% B in 30 minutes. t_R = 9.43 min; $[M+H]^+$ = 462.2 m/z

^{1}H-NMR (400 MHz, DMSO d6): δ 10.82 (s, 1H, H3(N) Trp^3), 8.15-8.00 (m, 4H, NH Aox^1, NH Gly^2, NH Trp^3, NH Lys^4), 7.61 (m, 2H, εCH_2(N) Lys^4), 7.59 (m, 1H, H7 Trp^3), 7.32 (m, 1H, H4 Trp^3), 7.16 (m, 1H, H2 Trp^3), 7.05 (m, H5 Trp^3), 6.98 (m, 1H, H6 Trp^3), 4.56 (m, 1H αCH Trp^3), 4.17 (m, 1H, αCH Lys^4), 3.85-3.63 (m, 4H, αCH_2 Aox^1, αCH_2 Gly^2), 3.15 and 2.96 (m, 2H, βCH_2 Trp^3), 2.76 (m, 2H, εCH_2 Lys^4), 2.68 and 2.34 (m, 2H, γCH_2 Lys^4), 1.56-1.47 (m, 4H, βCH_2 Lys^4, δCH_2 Lys^4).

Peptide A2

A2: H-Aox[1]-Gly[2]-Lys[3](Fmoc)-Trp[4]-Lys[5]-NH_2

"Fmoc-Rink Amide AM" with a theoretical loading of 0.65 mmol/g was used. For every step 3 eq of amino acid, Oxime and DIC were used, with respect to the moles of resin.

	MW (g/mol)	Moles (mmol)	Mass (mg)		Mass of Oxime (mg)	Volume of DIC (mL)	Volume of DIPEA (mL)
Fmoc-Rink-Amide AM + Lys[5] and Trp[4]	-	0.2275*	350*		-	-	-
Fmoc-Lys[3](Dde)-OH	574.6	0.6825	392.2	+	97.0	105.7	-
Fmoc-Gly[2]-OH	297.3	0.6825	202.9	+	97.0	105.7	-
Boc-Aox[1]-OH	191.2	0.6825	130.5	+	97.0	105.7	-
Fmoc-OSu	337.3	0.6825	230.6	+	-	-	233.7
Total					291.0	317.1	233.7

** in this case the indicated mass and moles are the theoretical once since the weighted resin has already some peptide bound to it*

The rest of the resin from **A1** was used for this synthesis. The rest of the ammino acids were added manually and following steps were done:

1. SWELLING
2. Gly[2] – COUPLING
3. FMOC REMOVAL
4. Aox[1] – COUPLING
5. FMOC REMOVAL
6. DDE REMOVAL
7. FMOC – COUPLING x 5
8. DCM DRYING
9. RELEASE OF 10 mg
10. RELEASE of all the resin

The obtained solid was purified by medium pressure flash chromatography and freeze-dried.

HPLC: solvent H_2O + 0.1%TFA. Gradient of 20-65% B in 30 minutes. t_R = 16.4 min

LC-MS: solvent H_2O + 0.1%TFA. Gradient of 5-95% B in 30 minutes. t_R = 15.7 min; $[M+H]^+$ = 812.3 m/z

^{1}H-NMR (400 MHz, DMSO d6): δ 10.80 (s, 1H, H3(N) Trp[4]), 8.12-6.96 (m, 21H, NH Aox[1], NH Gly[2], NH NH Lys[3], Trp[4], NH Lys[5], εCH$_2$(N) Lys[3], εCH$_2$(N) Lys[5], $H_{aromatic}$ Trp[4] and $H_{aromatic}$ Fmoc), 4.49-3.78 (m, 10H, αCH$_2$ Aox[1], αCH$_2$ Gly[2], αCH Lys[3], αCH Trp[4], αCH Lys[5], $H_{aliphatic}$ Fmoc), 3.19-2.93 (m, 2H βCH$_2$ Trp[4]), 2.74 (m, 4H, εCH$_2$ Lys[3], εCH$_2$ Lys[5]), 2.68 and 2.34 (m, 4H, γCH$_2$ Lys[3], γCH$_2$ Lys[5]), 1.54-1.47 (m, 8H, βCH$_2$ Lys[3], βCH$_2$ Lys[5], δCH$_2$ Lys[3], δCH$_2$ Lys[5]).

Tests with A1

KI addition: 8.5 mg of **A1** are dissolved in 6 mL of MilliQ water. The solution is split in two vials (3 mL each). To one of the vials 7.4 mg of KI are added.

*HPLC-**A1**:* Gradient 0-30% B in 30 minutes. $t_{R\text{-}A1}$ = 7.1 min; $t_{R\text{-}A1+40}$ = 19.6 min (same retention times for the HPLC performed right away and the day after)

*HPLC-**A1**+KI:* Gradient 0-30% B in 30 minutes. $t_{R\text{-}A1}$ = 7.1 min; $t_{R\text{-}A1+40}$ = 19.6 min (same retention times for the HPLC performed right away and the day after)

Vanillin conjugation: 10.2 mg of **A1** are dissolved in 3 mL of NaOAc/AcOH buffer (50mM, pH = 5) and 5 eq of vanillin are added to the solution.

*HPLC-**A1**:* Gradient of 5-95% B in 30 minutes. $t_{R\text{-}A1}$ = 3.9 min

HPLC-vanillin: Gradient of 5-95% B in 30 minutes. $t_{R\text{-vanillin}}$ = 14.2 min

HPLC-solution: Gradient of 5-95% B in 30 minutes. $t_{R\text{-product}}$ = 11.6 min; $t_{R\text{-vanillin}}$ = 14.2 min

The solution is stirred for one day and the HPLC are performed again:

HPLC-solution: Gradient 0-80% B in 30 minutes. $t_{R\text{-product}}$ = 16.0 min; $t_{R\text{-vanillin}}$ = 18.2 min

*HPLC-**A1**:* Gradient 0-80% B in 30 minutes. $t_{R\text{-}A1}$ = 6.0 min; $t_{R\text{-}A1+40}$ = 12.8 min

The two solutions above are co-eluted (150µL each):

HPLC: Gradient 0-80% B in 30 minutes. $t_{R\text{-}A1+40}$ = 12.8 min; $t_{R\text{-product}}$ = 16.0 min

Synthesis of antimicrobial peptides

Peptide C2

C2: H-Cys[1]-Lys[2]-Arg[3]-Leu[4]-Lys[5]-Lys[6]-Ile[7]-Gly[8]-Lys[9]-Val[10]-Leu[11]-Lys[12]-Trp[13]-Ile[14]-NH_2

"Fmoc-Rink Amide AM" with a theoretical loading of 0.65 mmol/g was used. For every step until the Lys[2] 3 eq of amino acid, HBTU, HOBt and 6eq of DIPEA were used, with respect to the moles of resin. Then 3 eq of amino acid, Oxime and DIC were used, with respect to the moles of resin.

	MW (g/mol)	Moles (mmol)	Mass (mg)		Mass of Oxime (mg)	Volume of DIC (mL)
Fmoc-Rink-Amide AM + PMAP(12-24)	-	0.1625	250		-	-
Fmoc-Lys(Boc)-OH	468.5	2.925	1484.6		-	-
Fmoc-Arg(Pbf)-OH	648.8	0.975	790.7		-	-
Fmoc-Leu-OH	353.4	1.950	775.3		-	-
Fmoc-Ile-OH	353.4	1.462	603.0		-	-
Fmoc-Gly-OH	297.3	0.4875	217.4		-	-
Fmoc-Val-OH	339.4	0.975	413.6		-	-
Fmoc-Trp(Boc)-OH	526.6	0.975	641.8		-	-
HBTU tot	379.3	9.750	3790.6		-	-
HOBt*H_2O tot	153.1	9.750	1530.0		-	-
Fmoc-Cys(Trt)-OH	585.7	0.2438	142.8	+	34.6	37.7

The synthesis of the 2-14 fragment was performed automatically following these steps:

1. SWELL
2. DEPR + WASH
3. Ile^{14} – COUP + WASH x2
4. DEPR + WASH
5. Trp^{13} – COUP + WASH x2
6. DEPR + WASH
7. Lys^{12} – COUP + WASH
8. DEPR + WASH
9. Leu^{11} – COUP + WASH x2
10. DEPR + WASH
11. Val^{10} – COUP + WASH x2
12. DEPR + WASH
13. Lys^{9} – COUP + WASH
14. DEPR + WASH
15. Gly^{8} – COUP + WASH
16. DEPR + WASH
17. Ile^{7} – COUP + WASH
18. DEPR + WASH
19. Lys^{6} – COUP + WASH
20. DEPR + WASH
21. Lys^{5} – COUP + WASH
22. DEPR + WASH
23. Leu^{4} – COUP + WASH x2
24. DEPR + WASH
25. Arg^{3} – COUP + WASH x2
26. DEPR + WASH
27. Lys^{2} – COUP + WASH x2
28. DEPR + WASH
29. WASH DCM

Then the resin was split in two: the first 125 mg were used for this synthesis; the rest was used for the synthesis of **A3**. Cys^{1} was added manually according to the following steps:

1. SWELLING
2. Cys^{1} – COUPLING
3. FMOC REMOVAL
4. DCM DRYING
5. RELEASE of all the resin

The obtained solid was purified by medium pressure flash chromatography and freeze-dried.

Yield: 22%

HPLC: solvent H_2O + 0.1% TFA. Gradient of 0-50% B in 30 minutes. t_R = 15.3 min

LC-MS: solvent H_2O + 0.1% TFA. Gradient of 5-95% B in 30 minutes. t_R = 11.8 min; $[M+H]^+$ = 1713.2 m/z

Peptide A3

A3: H-Aox^{1}-Lys^{2}-Arg^{3}-Leu^{4}-Lys^{5}-Lys^{6}-Ile^{7}-Gly^{8}-Lys^{9}-Val^{10}-Leu^{11}-Lys^{12}-Trp^{13}-Ile^{14}-NH_2

"Fmoc-Rink Amide AM" with a theoretical loading of 0.65 mmol/g was used. For every step until the Lys^{2} 3 eq of amino acid, HBTU, HOBt and 6eq of DIPEA were used, with respect to the moles of resin. Then 3 eq of amino acid, Oxime and DIC were used, with respect to the moles of resin.

	MW (g/mol)	Moles (mmol)	Mass (mg)		Mass of Oxime (mg)	Volume of DIC (mL)
Fmoc-Rink-Amide AM + PMAP(12-24)	-	0.0813*	125*		-	-
Boc-Aox-OH	191.2	0.2438	46.6	+	34.6	37.7

** in this case the indicated mass and moles are the theoretical one since the weighted resin has already some peptide bound to it*

The rest of the resin from **C2** was used. Aox[1] was added manually according to the following steps:

6. SWELLING
7. Aox[1] – COUPLING
8. DCM DRYING
9. RELEASE of all the resin

Yield: 95% (before the purification)

HPLC: solvent H_2O + 0.1% TFA. Gradient of 0-50% B in 30 minutes. t_R = 16.4 min

LC-MS: solvent H_2O + 0.1% TFA. Gradient of 5-95% B in 30 minutes. t_R = 12.3 min; $[M+H]^+$ = 1683.2 m/z

5.5 COTTON FUNCTIONALIZATION

Comparison of different types of cotton

EXP-1: **C1** was dissolved in 2.5 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of about 0.15 mM, then 1eq of TCEP, with respect to the peptide, and the cotton were added to the mixture. The reaction took place in a quartz cuvette (optical path 1cm) and was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance at 280 nm. This was done for A-, B- and C-cotton.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
A-cot + C1	0.0119	0.140	29.5	0.0106	2 hours
B-cot + C1	0.0131	0.154	29.6	0.0113	2 hours
C-cot + C1	0.042	0.16	9.5	0.040	1 day

EXP-2: **C1** was dissolved in 40 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of 0.17 mM, then 1eq of TCEP, with respect to the peptide, and the cotton were added to the mixture. The reaction was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance at 280 nm. This was done for A-, B- and C-cotton.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
A-cot + C1	0.436	0.170	16.5	0.162	3-6 days
B-cot + C1	0.632	0.170	11.4	0.150	3-5 days
C-cot + C1	0.626	0.170	11.5	0.145	3-5 days

Once the reaction is completed, the cottons were removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a desiccator. Then the Kaiser and Schiff tests were performed. All the Kaiser

tests resulted blue, while the cottons after the Schiff tests from EXP-1 were slight pink and the ones from EXP-2 did not change color.

Effect of concentration

EXP-3: **C1** was dissolved in 25 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of 2.18 mM, then 1eq of TCEP, with respect to the peptide, and the A-cotton were added to the mixture. The reaction was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance at 280 nm.

EXP-4: **C1** was dissolved in 100 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of 1.05 mM, then 1eq of TCEP, with respect to the peptide, and the B-cotton were added to the mixture. The reaction was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance at 280 nm.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
EXP-3	0.987	2.18	55.2	0.160	1 day
EXP-4	1.21	1.05	86.7	0.100	1 day

EXP-5: **A1** was dissolved in 50 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of 1.70 mM, the B-cotton was added to the mixture. The reaction was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance at 280 nm.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
EXP-5	2.04	1.70	41.6	0.126	1 day

Once the reaction is completed, the cottons were removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a desicator. Then the Kaiser and Schiff tests were performed. In all the experiments the Kaiser test resulted blue and the addition of the Schiff reagent did not induce a change in the color of the cotton.

Re-use of the peptide solution

EXP-6: A-cotton and 1eq TCEP, with respect to the peptide, were added to 23 mL of the **C1** solution used in EXP-3. The reaction was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance at 280 nm.

EXP-7: B-cotton and 1eq TCEP, with respect to the peptide, were added to the **C1** solution used in EXP-4. The reaction was monitored through UV-vis and the solution was stirred until there was no more decreasing

in absorbance at 280 nm. This was done for 3 times and every time the taken volume was 5 mL less than the previous experiment.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
EXP-6	1.93	1.84	22.0	0.283	1 day
EXP-7 1st re-use	2.00	0.950	45.1	0.105	1 day
EXP-7 2nd re-use	1.94	0.908	42.0	0.213	1 day
EXP-7 3rd re-use	1.95	0.796	37.7	0.187	1 day

EXP-8: B-cotton was added to the **A1** solution used in EXP-5. The reaction was monitored through UV-vis and the solution was stirred until there was no more decreasing in absorbance at 280 nm e. This was done for 2 times and every time the taken volume was 5 mL less than the previous experiment.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
EXP-8 1st re-use	1.90	1.59	34.7	0.093	1 day
EXP-8 2nd re-use	2.16	1.50	27.3	0.090	1 day

Once the reaction is completed, the cottons were removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Kaiser and Schiff tests were performed. All the Kaiser tests resulted blue and the addition of the Schiff reagent did not induce a change in the color of the cotton.

Addition of TCEP

EXP-9: B-cotton was added to 80 mL of the **C1** solution used for the 3rd re-use in EXP-7. The reaction was monitored through UV-vis and until there was no more decreasing in absorbance at 280 nm. Then, 1eq of TCEP, with respect to the peptide, was added to the solution; this was done 3 times.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
EXP-9	2.30	0.725	25.5	0.620	9 days

Once the reaction is completed, the cotton was removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Kaiser and Schiff tests were performed. The Kaiser test resulted blue and the addition of the Schiff reagent did not induce a change in the color of the cotton.

Re-use of the oxidation mixture

EXP-10: **C1** was dissolved in 60 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of 1.07 mM, then 1eq of TCEP, with respect to the peptide, was added to the mixture. The solution was divided in 3 vials with 15 mL each and the A-cottons from the 1st, 2nd and 3rd oxidation (see 5.3) were added. The reactions were monitored through UV-vis. The solutions were stirred until there was no more decreasing in absorbance at 280 nm.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
A-cot-1stox + C1	1.36	1.07	11.8	0.147	1 day
A-cot-2ndox + C1	1.53	1.07	10.5	0.117	1 day
A-cot-3rdox + C1	1.34	1.07	12.0	0.069	1 day

Once the reaction is completed, the cottons were removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Kaiser and Schiff tests were performed. All the Kaiser tests resulted blue and the addition of the Schiff reagent did not induce a change in the color of the cotton.

Use of Fmoc for loading determination

EXP-11: 20.2 mg of **A2** were dissolved in 15 mL of a NaOAc/AcOH buffer (50mM, pH = 5), 5.2 mg of B-cotton were added to the mixture. The reaction was monitored through UV-vis. The solution was stirred until there was no more decreasing in absorbance (3 days) . After that the cotton was removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Fmoc group was removed from the cotton (as described in 5.4), the Fmoc/piperidine solution was collected in a 10 mL graduated flask and made up to the mark with DMF. The absorbance of the so obtained solution was measured at 289.8 nm and a loading of 0.0794 $mmol_{peptide}/g_{cotton}$ was calculated. At the end, the cotton washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Kaiser and Schiff tests were performed. The Kaiser test resulted blue and the Schiff reagent made the cotton slightly pink

Conjugation of the antibacterial peptides

EXP-12: **C2** was dissolved in 8 mL of NaOAc/AcOH buffer (50mM, pH = 5) in a concentration of 1.46 mM, then 1eq of TCEP, with respect to the peptide, and A-cotton were added to the mixture. The reaction was

monitored through UV-vis and the solution was stirred until there was no more decreasing in absorbance at 280 nm.

EXP-13: : B-cotton and 1eq of TCEP, with respect to the peptide, were added to 7mL of the **C2** solution used in EXP-12. The reaction was monitored through UV-vis and the solution was stirred until there was no more decreasing in absorbance at 280 nm.

EXP-14: The solution from EXP-13 was freeze-dried and the obtained solid was dissolved in 4mL of OAc/AcOH buffer (50mM, pH = 5), then 1eq of TCEP, with respect to the peptide, and B-cotton were added to the mixture. The reaction was monitored through UV-vis and the solution was stirred until there was no more decreasing in absorbance at 280 nm.

Sample	USED $mmol_{peptide}/g_{cotton}$	Concentration of peptide (mM)	Mass of cotton (mg)	LOADING ($mmol_{peptide}/g_{cotton}$)	Time
EXP-12	0.525	1.46	22.2	0.0386	1 day
EXP-13	0.570	1.38	16.9	0.0416	1 day
EXP-14	0.328	2.35	28.7	0.0187	1 day

Once the reaction is completed, the cottons were removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Kaiser and Schiff tests were performed. Cottons from EXP-12 to EXP-14 were used to do the biologic analysis.

Release of the peptide from cotton

The release of peptide **C1** from A-cotton (from EXP-3) was first tested in 70% EtOH in MilliQ water at a room temperature of ca. 25°C. To do so, 3mL of solution were added, in a cuvette (optical path 1 cm), to the functionalized cotton. The solution was stirred for 5 hours and its absorbance at 280 nm checked at 0 min, and 1, 2, 5 hours.

Solution	Peptide	Mass $_{functionalized\ cotton}$ (mg)	Peptide loss after 5h
70% EtOH	C1	20.0	2%

The release of peptides **C1** and **A1** from B-cotton (from EXP-4 and EXP-5) was investigated at pH 3 (50mM sodium citrate/citric acid buffer), 7 (PBS buffer) and 10 (50mM sodium carbonate/sodium bicarbonate buffer), in HCl 3M and 70% EtOH in MilliQ water at a room temperature of ca. 30°C. To do so, 2.5mL of the chosen solution were added, in a cuvette (optical path 1 cm), to the functionalized cotton. The solutions were stirred for 3 days and its absorbance at 280 nm checked at 0 min, 2, 4, 6 hours, 1, 2 and 3 days.

Solution	Peptide	Mass $_{\text{functionalized cotton}}$ (mg)	Peptide loss after 3 days
pH 3	C1	11.2	18%
	A1	8.3	10%
pH 7	C1	5.5	<4%
	A1	6.9	<3%
pH 10	C1	10.9	6%
	A1	8.5	9%
HCl 3M	C1	9.7	33%
	A1	9.0	> 40%
70% EtOH	C1	5.6	10%
	A1	8.5	4%

The peptide loss was calculated as the ratio between the released moles and the total moles bound to the cotton. After the reactions took place, the cottons were removed from the solution, washed with water (3mL x 7) and acetone (3mL x 7) and dried in a drier. Then the Kaiser and Schiff test were performed. All the Kaiser tests resulted blue while the Schiff tests did not change the color of the cotton, except for **A1** in HCl 3M and **C1** at pH 3.

Biologic analysis

All biological experiments have been performed by research group of Prof. M. Scocchi at the University of Trieste. Briefly, for the biofilm test small cotton circles were soaked in the culture medium (1mL MH) with bacteria (1x106 Cfu/mL A. Baumanni) for 24 h, then bacterial growth was determined. For cell viability tests cotton samples were plunged for 4 hours in 1mL of culture medium (MH) with cell viability indicator (Resazurin) at 37°C to assess bacterial biofilm growth.

6. BIBLIOGRAPHY

1. Gpyal, S., et al., *Bacterial Contamination of Medical Providers' White Coats and Surgical Scrubs: A Systematic Review.* American Journal of Infection Control, 2019. **47**: p. 994-1001.
2. Zhang, Y.Y., et al., *Durable Antimicrobial Cotton Textiles Modified with Inorganic Nanoparticles.* Cellulose, 2016. **23**: p. 2791-2808.
3. Granados, A., R. Pleixats, and A. Vallribera, *Recent Advances on Antimicrobial and Anti-Inflammatory Cotton Fabrics Containing Nanostructures.* Molecules (Basel, Switzerland), 2021. **26**(10): p. 3008.
4. Elshafei, A. and H.T. El-Zanfaly, *Application of Antimicrobials in the Development of Textiles.* Asian Journal of Applied Sciences 2011. **4**: p. 585-595.
5. Reed, R., et al., *Potential Environmental Impacts and Antimicrobial Efficacy of Silverand Nanosilver-Containing Textiles.* Environmental Science and Technology, 2016. **50**: p. 4018-4026.
6. Scapin, S., *Ancoraggio di Peptidi Antimicrobici su Superficie di Cotone Mediante Legame Tiazolidinico e Ossazolidinico* Msc Thesis, 2018.
7. Scapin, S., et al., *Sustainable, Site-Specific Linkage of Antimicrobial Peptides to Cotton Textiles.* Macromolecular bioscience, 2020. **2000199**.
8. Büyükkiraz, M.E. and Z. Kesmen, *Antimicrobial Peptides (AMPs): A Promising Class of Antimicrobial Compounds.* Journal of Applied Microbiology, 2021. **132**: p. 1573–1596.
9. Spears, R.J. and M.A. Fascione, *Site-selective Incorporation and Ligation of Protein Aldehydes.* Organic & Biomolecular Chemistry, 2016. **14**(32): p. 7622-7638.
10. Fang, D.D., *Cotton Fiber: Physics, Chemistry and Biology.* Book, 2018.
11. P.J., W., et al., *Cotton Fiber: Chemistry and Technology.* Book, 2006.
12. Haigler, C.H., et al., *Cotton Fiber: a Powerfull Single-cell Model for Cell Wall and Cellulose Research* Frontiers in plant science, 2012. **3**.
13. Dorchia, M., *Handbook of Natural Fibres.* Book, 2012.
14. Pinho, E. and G. Soares, *Functionalization of Cotton Cellulose for Improved Wound Healing.* Journal of Materials Chemistry B, 2018. **6**: p. 1887-1898.
15. Payen, A. and C.R. Hebd., *Mémoire sur la Composition du Tissu Propre des Plantes et du Ligneux.* Seances Acad., 1838. **7**.
16. Staudinger, H., *Über Polymerisation.* Ber. Dtsch. Chem. Ges., 1920. **53**: p. 1073 – 1085.
17. Klemm, D., et al., *Cellulose: Fascinating Biopolymer and Sustainable Raw Material.* Polymer science 2005. **44**: p. 3358-3393.
18. Li, T., et al., *Developing Fibrillated Cellulose as a Sustainable Technological Material.* Nature, 2020. **590**: p. 47-56.

19. Okano, T. and A. Sarko, *Mercerization of Cellulose. 11. Alkali-Cellulose Intermediates and a Possible Mercerization Mechanism.* Journal of Applied Polymer Science, 1997. **30**: p. 325-332.
20. Coseri, S., et al., *Oxidized Cellulose—Survey of the Most Recent Achievements.* carbohydrate Polymers, 2013. **93**: p. 207-215.
21. Semmelhack, M.F., C.S. Chou, and D.A. Cortes, *Nitroxyl-mediated Electrooxidation of Alcohols to Aldehydes and Ketones.* Journal of the American Chemical Society, 1983. **105**: p. 4492-1194.
22. Jiang, J., et al., *Rate-Limited Reaction in TEMPO/Laccase/O2 Oxidation of Cellulose.* Macromolecular rapid communications 2021. **42**.
23. Yoshida, H., *Chemistry of Lacquer (urushi).* Journal of Chemical Society, 1883. **43**: p. 472–486.
24. Kunamneni, A., et al., *Engineering and Applications of Fungal Laccases for Organic Synthesis.* Microbial Cell Factory, 2008. **7**.
25. Mayer, A.M. and R.C. Staples, *Laccase: New Functions for an Old Enzyme.* Photochemistry, 2002. **60**: p. 551-565.
26. Viikari, L., K. Kruus, and J. Buchert, *Method for Modification of Cellulose.* European Pat WO9923117, 2000.
27. Tromp, S.A., et al., *Mechanism of Laccase–TEMPO-Catalyzed Oxidation of Benzyl Alcohol.* chemical catalysis, 2010. **2**: p. 827-833.
28. Aracri, E., C. Valls, and V. Teresa, *Paper Strength Improvement by Oxidative Modification of Sisal Cellulose Fibers with Laccase–TEMPO System: Influence of the Process Variables.* Carbohydrate Polymers, 2012. **88**: p. 830-837.
29. Jones, S.M. and E.I. Solomon, *Electron Transfer and Reaction Mechanism of Laccases.* Cellular and Molecular Life Science, 2015. **75**: p. 869-883.
30. Aracri, E. and T. Vidal, *Enhancing the Effectiveness of a Laccase–TEMPO Treatment has a Biorefining Effect on Sisal Cellulose Fibres.* Cellulose, 2012. **19**: p. 867-877.
31. Arends, I.W.C.E., Y. Li, and R.A. Sheldon, *Stabilities and Rates in the Laccase/TEMPO Catalyzed Oxidation of Alcohols.* Biocatalysis and Biotransformation, 2006. **24**: p. 443-448.
32. Sheldon, R.A. and S. van Pelt, *Enzyme Immobilisation in Biocatalysis: Why, What and How.* Chemical Society Reviews, 2013. **42**: p. 6223-6235.
33. Robins, J.H., G.D. Abrams, and J.A. Pincock, *The Structure of Schiff Reagent Aldehyde Adducts and the Mechanism of the Schiffreaction as Determined by Nuclear Magnetic Resonance Spectroscopy* Canadian Journal of Chemistry, 1980. **58**: p. 339-347.
34. Kent, S.B.H., *Total Chemical Synthesis of Proteins.* Chemical Society Reviews, 2009. **38**: p. 338-351.
35. Schnolzer, M. and S.B.H. Kent, *Constructing Proteins by Dovetailing Unprotected Synthetic Pptides: Backbone-Engineered HIV Proteas.* Science 1992. **256**: p. 221-225.
36. Tiefenbrunn, T.K. and P.E. Dawson, *Chemoselective Ligation Techniques: Modern Applications of Time-Honored Chemistry.* Peptide Science, 2010. **94**: p. 95-106.

37. Ramakers, B.E.I., J.C.M. van Hest, and D.W.P.M. Löwik, *Molecular Tools for the Construction of Peptide-based Materials.* Chemical Society Reviews, 2014. **43**(8): p. 2743-2756.
38. Dawson, P.E., et al., *Synthesis of Proteins by Native Chemical Ligation.* Science, 1994. **266**: p. 776-779.
39. Hackenberger, C.P.R. and D. Schwarzer, *Chemoselective Ligation and Modification Strategies for Peptides and Proteins.* Angewandte Chemie International Edition, 2008. **47**: p. 10030-10074.
40. Kulkarni, S.S., et al., *Rapid and Efficient Protein Synthesis Through Expansion of the Native Chemical Ligation Concept.* Nature Reviews Chemistry, 2018. **2**: p. 0122.
41. Wan, Q. and S.J. Danishefsky, *Free-radical-based, Specific Desulfurization of Cysteine: a Powerful Advance in the Synthesis of Polypeptides and Glycopolypeptides.* Angew. Chem. , 2007. **46**: p. 9248–9252.
42. Englebretsen, D.R., et al., *A Novel Thioether Linker: Chemical Synthesis of a HIV-1 Protease Analogue by Thioether Ligation.* Tetrahedron letters, 1995. **36**: p. 8871-8874.
43. Baca, M., et al., *Chemical Ligation of Cysteine-Containing Peptides: Synthesis of a 22 kDa Tethered Dimer of HIV-1 Protease.* Journal of American Chemical Society, 1995. **117**: p. 1881-1887
44. Henkel, M., N. Rockerndorf, and A. Frey, *Selective and Efficient Cysteine Conjugation by Maleimides in the Presence of Phosphine Reductants.* Bioconjugate Chemistry, 2016. **27**: p. 2260−2265.
45. Tornoe, C.W., C. Christensen, and M. Meldal, *Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides.* Journal of Organic Chemistry, 2002. **67**: p. 3057-3064.
46. Saxton, E. and C.R. Bertozzi, *Cell Surface Engineering by a Modified Staudinger Reaction.* Science, 2000. **287**: p. 2007-2010
47. Liu, C.-F. and J.P. Tam, *Chemical Ligation Approach To Form a Peptide Bond between Unprotected Peptide Segments. Concept and Model Study.* Journal of American Chemical Society, 1994. **116**: p. 4149-4153.
48. Tam, J.P., et al., *Specificity and Formation of Unusual Amino Acids of an Amide Ligation Strategy for Unprotected Peptides.* International Journal of Peptide and Protein Research, 1994. **45**: p. 209–216.
49. Rose, K., *Facile Synthesis of Homogeneous Artificial Proteins.* Journal of American Chemical Society, 1994. **116**: p. 30-33.
50. King, T.P. and L. Z.S.W., *Preparation of Protein Conjugates via Intermolecular Hydrazone Linkage.* Biochemistry, 1986. **25**: p. 5774-5779.
51. Bermejo-Velasco, D., et al., *Thiazolidine Chemistry Revisited: a Fast, Efficient and Stable Click-Type Reaction at Physiological pH.* Chemical Communications, 2018. **54**: p. 12507--12510.
52. Meyer, V. and A. Janny, *Ueber Stickstoffhaltige Acetonderivate.* Ber. Dtsch. Chem. Ges., 1882. **15**: p. 1164−1167.
53. Fischer, E., *Ueber die Hydrazone.* Ber. Dtsch. Chem. Ges. , 1888. **21**: p. 984−988.

54. Kolmel, D.K. and E.T. Kool, *Oximes and Hydrazones in Bioconjugation: Mechanism and Catalysis.* Chemical Reviews, 2017. **117**: p. 10358-10376.
55. Shao, J. and T. J.P., *Unprotected Peptides as Building Blocks for the Synthesis of Peptide Dendrimers with Oxime, Hydrazone, and Thiazolidine Linkages.* Journal of American Chemical Society, 1995. **117**: p. 3893-3899.
56. Sanchez, A. and A. Vazquez, *Bioactive Peptides: A Review.* Food Quality and safety 2017. **1**: p. 29-46.
57. Dubos, R.J., *Studies on a Bactericidal Agent Extracted from a Soil Bacillus: I. Preparation of the Agent. Its Activity in Vitro.* The Journal of experimental medicine, 1939. **70**: p. 1-10.
58. Dubos, R.J., *Studies on a Bactericidal Agent Extracted from a Soil Bacillus: II. Protective Effect of the Bactericidal Agent Against Experimental Pneumococcus Infections in Mice.* The Journal of experimental medicine, 1939. **70**: p. 11-17.
59. R.D., H. and D. R.J., *Fractionation of the Bactericidal Agent from Cultures of a Soil Bacillus.* Journal of Biological Chemistry, 1940. **132**: p. 791-792.
60. Ageitos, J.M., et al., *Antimicrobial Peptides (AMPs): Ancient Compounds that Represent Novel Weapons in the Fight Against Bacteria.* Biochemical Pharmacology, 2017. **133**: p. 117-138.
61. Freitas, E.D., et al., *Antimicrobial Peptides and Their Potential Application in Antiviral Coating Agents.* Colloids and surfaces: Biointerfaces, 2022. **avaiable online**.
62. Wang, J., et al., *Antimicrobial Peptides: Promising Alternatives in the Post Feeding Antibiotic Era.* Medicinal research reviews, 2019. **39**: p. 831-859.
63. Costa, F., et al., *Covalent Immobilization of Antimicrobial Peptides (AMPs) onto Biomaterial Surfaces.* Acta Biomaterialia, 2011. **7**: p. 1431-1440.
64. Ciumac, D., et al., *Membrane Targeting Cationic Antimicrobial Peptides.* Journal of Colloid and Interface Science 2019. **537**: p. 163-185.
65. Pajerski, W., et al., *Attachment Efficiency of Gold Nanoparticles by Gram-positive and Gram-negative Bacterial Strains Governed by Surface Charges.* Journal of Nanoparticles research 2019. **21**: p. 1-12.
66. Yang, L., et al., *Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores.* Biophysical Juournal, 2001. **81**: p. 1475–1485.
67. Gazit, E., et al., *Structure and Orientation of the Mammalian Antibacterial Peptide Cecropin P1 within Phospholipid Membranes.* Journal of Molecular Biology, 1996. **258**: p. 860–870.
68. Gagnon, M.G., et al., *Structures of Proline-rich Peptides Bound to the Ribosome Reveal a Common Mechanism of Protein Synthesis Inhibition.* Nucleic Acids Research, 2016 **44**: p. 2439– 2450.
69. Park, C.B., H.S. Kim, and S.C. Kim, *Mechanism of Action of the Antimicrobial Peptide Buforin II: Buforin II Kills Microorganisms by Penetrating the Cell Membrane and Inhibiting Cellular Functions.* Biochemical and Biophysical Research Communications, 1998. **244**: p. 253– 257.
70. Otvos, L., et al., *Interaction Between Heat Shock Proteins and Antimicrobial Peptides.* Biochemistry, 2000. **39**: p. 14150– 14159.

71. Pletzer, D., S.R. Coleman, and R.E. Hancock, *Anti- biofilm Peptides as a New Weapon in Antimicrobial Warfare.* Current Opinion in Microbiology, 2016. **33**: p. 35-40.
72. Zhang, G., C. Ross, and F. Blecha, *Porcine Antimicrobial Peptides: New Prospects for Ancient Molecules of Host Defense.* Veterinary research 1999. **31**: p. 277-296.
73. Schhnstra, M.R., et al., *Cathelicidins PMAP-36, LL-37 and CATH-2 are Similar Peptides with Different Modes of Action.* Scintific reports, 2019. **9**: p. 1-12.
74. Storici, P., et al., *Chemical Synthesis and Biological Activity of a Novel Antibacterial Peptide Deduced From a Pig Myeloid cDNA* FEBS letters, 1994. **337**: p. 303-307.
75. Scocchi, M., et al., *Structural Aspects and Biological Properties of the Cathelicidin PMAP-36.* The FEBS Journal, 2005. **272**: p. 4398-4406.
76. Lv, Y., et al., *Antimicrobial Properties and Membrane-Active Mechanism of a Potential α-Helical Antimicrobial Derived from Cathelicidin PMAP-36.* PloS One, 2014. **9**.
77. Lyu, Y., et al., *Antimicrobial activity, Improved Cell Selectivity and Mode of Action of Short PMAP-36-derived Peptides Against Bacteria and Candida.* Scintific reports, 2016. **6**: p. 1-12.
78. Merrifield, R.B., *Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide.* Journal of the American Chemical Society, 1963. **85**: p. 2149-2154.
79. Amblard, M., et al., *Methods and Protocols of Modern Solid Phase Peptide Synthesis.* Molecular Biotechnology, 2006. **33**: p. 239-254.
80. Bachem, *Tips and Tricks for Solid Phase Peptide Synthesis from the Experts at Bachem.* 2018.
81. Chan, W.C. and P.D. White, *Fmoc Solid Phase Peptide Synthsis - A practical Approach.* Book, 2000.
82. Sherrington, D.-C.-. *Preparation, Structure and Morphology of Polymer Supports.* Chemical Communications, 1998. **21**.
83. Atherton, E., et al., *A Mild Procedure for Solid Phase Peptide Synthesis: Use of Fluorenylmethoxycarbonylamino -acids.* Journal of the Chemical Society, Chemical Communications, 1978. **13**: p. 537-539.
84. Isidro-Llobet, A., M. Alvarez, and F. Albericio, *Amino Acid-Protecting Groups.* Chemical Reviews, 2009. **109**: p. 2455-2504.
85. Valeur, E. and M. Bradley, *Amide Bond Formation: Beyond the Myth of Coupling Reagents.* Chemical Society Reviews, 2008. **38**: p. 606-631.
86. Al-Warhi, T., H.M.A. Al-Hazimi, and A. El-Faham, *Recent Development in Peptide Coupling Reagents.* Journal of Saudi Chemical Society, 2012. **16**: p. 97-116.
87. Carpino, L.A., A. El-Faham, and F. Albericio, *Racemization Studies During Solid-phase Peptide Synthesis Using Azabenzotriazole-based Coupling Reagents.* Tetrahedron letters, 1994. **35**: p. 2279–2282
88. Subirós-Funosas, R., et al., *Oxyma: An Efficient Additive for Peptide Synthesis to Replace theBenzotriazole-Based HOBt and HOAt with a Lower Risk of Explosion.* Chemistry – A European Journal, 2009. **15**: p. 9394-9403.

89. E. Kaiser, e.a., Analytical Biochemistry, 1970. **34**: p. 595-598.
90. Sarin, V., et al., *Quantitative Monitoring of Solid-Phase Peptide Synthesisby the Ninhydrin Reaction.* Analytical Biochemistry, 1981. **117**: p. 147-157.
91. Pace, C.N., et al., *Hoe to Measure and Predict the Molar Absorption Coefficient of a Protein.* Protein Science, 1995. **4**: p. 2411-2423.
92. Eissler, S., et al., *Substitution Determination of Fmoc-substituted Resins at Different Wavelengths.* Journal of Peptide Science, 2017. **23**: p. 757-762.
93. Puella, P.K., et al., *Fluorescence-based Detection of Thiols in Vitro and in Vivo Using Dithiol Probes.* Analytical Biochemistry, 2006. **352**: p. 265-273.
94. Socrates, G., *Infrared and Raman characteristic group frequencies.* Book, 2004. **John Wiley & Sons**.
95. Vaideki, K., et al., *A Study on the Antimicrobial Efficacy of RF Oxygen Plasma and Neem Extract Treated Cotton Fabrics.* Applied Surface Science, 2007. **253**: p. 7323-7329.
96. Hazam, P.K., R. Goyal, and V. Ramakrishnanet, *Peptide Based Antimicrobials: Design Strategies and Therapeutic Potential.* Progress in Biophysics and Molecular Biology, 2019. **142**: p. 10– 22.

RINGRAZIAMENTI

Concludo questo elaborato ringraziando in primo luogo la prof. Cristina Peggion per l'opportunità di svolgere la tesi preso il suo laboratorio di ricerca, ma anche per la guida e i consigli durante questi mesi. Ringrazio inoltre tutte le persone del gruppo di ricerca per la calda accoglienza in laboratorio.

Ringrazio il mio controrelatore prof. Stefano Casalini per la disponibilità durante gli incontri e l'interesse mostrato nei confronti del mio lavoro.

Grazie a colleghi amici sia dell'università che del collegio per aver condiviso con me le gioie e le tristezze del mio percorso universitario. Senza di voi questa esperienza non sarebbe stata vissuta appieno.

Ringrazio infine la mia famiglia per avermi sostenuto in questi anni. In particolare, un grazie ai miei genitori per avermi supportato qualsiasi fossero le mie scelte e a mio fratello per aver sempre creduto in me. Un grazie perché senza di voi non sarei mai arrivata fino a qui.