Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron 63 (2007) 9033–9047
Tetrahedron

Tetrahedron report number 809

Histidinoalanine: a crosslinking amino acid

Carol M. Taylor* and Weihua Wang

Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA

Received 14 May 2007
Available online 2 June 2007

Contents

1. Introduction

As the name suggests, this bis-amino acid is derived literally by covalently linking the N_{im} of a histidine residue to Cβ of an alanine residue. Two regioisomers (τ-HAL and π-HAL) are possible (Fig. 1), depending on which nitrogen of the imidazole is linked to the 'Ala' residue.

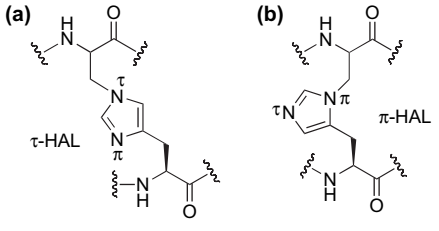


Figure 1. Structure of HAL regioisomers.

The histidinoalanine (HAL) protein crosslink was proposed by Finley and Friedman in 1977.[1] A few years later, Fujimoto et al. were the first to isolate HAL from a protein and characterize the molecule.[2] Both isomers have been identified from a range of sources:

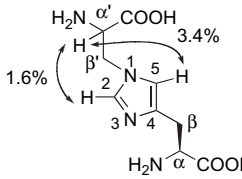
- milk products that have been heated and/or treated with alkali;
- human tissues, including: connective tissue, bone, dentin, and eye cataracts;
- phosphoproteins of bivalve mollusks; and
- the theonellamides: a family of bicyclic dodecapeptides; the first example of this crosslink in a well-defined sequence.

In the early 1980s Fujimoto et al. published the isolation and characterization of τ-HAL.[2] Almost simultaneously, Sass and Marsh reported NMR and mass spectrometric data for both τ- and π-regioisomers.[3] Proton NMR data for the τ-isomer are reported in Table 1. Sass and Marsh state that 'chemical shifts are very sensitive to pD and care must be taken in the comparison of spectra recorded at different

* Corresponding author. E-mail: cmtaylor@lsu.edu

0040–4020/$ - see front matter
doi:10.1016/j.tet.2007.05.114

Table 1. ^{1}H NMR data for τ-HAL. Double-headed arrows and percentages refer to NOEs reported by Fujimoto et al.[2]



δ/ppm[a] (J/Hz)	δ/ppm[b] (J/Hz)	Assignment (integral)
3.38 (dd, 16, 7)	3.16 (d, 9)	Hβ[a] (1H)
3.42 (dd, 16, 7)	3.18 (d, 5)	Hβ[b] (1H)
4.23 (t, 7)	3.96–4.19 (m)	Hα (1H)
4.38 (t, 7)		Hα′ (1H)
4.78[c]	4.55 (d, 5)	Hβ′ (2H)
7.54 (s)	7.09 (s)	H5 (1H)
8.86 (s)	7.71 (s)	H2 (1H)

[a] Ref. 2 (360 MHz, D_2O, referenced to TSP as an external standard).
[b] Ref. 3 (90 MHz, D_2O, pD7, referenced to TSP as an internal standard).
[c] Overlapped with water signal; determined unambiguously via decoupling experiment.

values of pD'.[4] Since Fujimoto et al. do not discuss pD, we can probably conclude that this accounts for the significant differences in chemical shift and peak shape. Indeed, on lowering the pD to 5.5, Sass and Marsh state that the Hα and Hα′ signals were resolved into two triplets.

The proton NMR data for the π-isomer, as reported by Sass and Marsh,[3,4] are summarized in Figure 2. More recently, Henle et al. reported ^{1}H NMR data for two synthetic compounds that they presumed to be τ-HAL and π-HAL.[5] Their spectra were recorded in DMSO-d_6; they do not give the field strength at which they ran the experiments. Assignments were based on correlations in the TOCSY spectrum.

Fujimoto et al. reported NOEs between Hα′ (of the 'Ala' residue) and both H2 and H5 of the imidazole (Table 1); this was taken as evidence for the τ-regioisomer.[2] Henle et al. reported similar NOE's for the τ-isomer and a single positive NOE between Hβ′ and H2 for the π-isomer.[5]

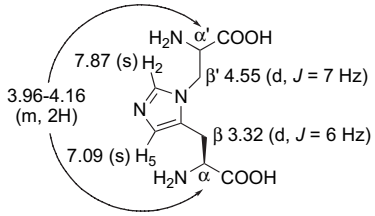


Figure 2. ^{1}H NMR data (D_2O, pD 7, 90 MHz) of π-HAL.[3,4]

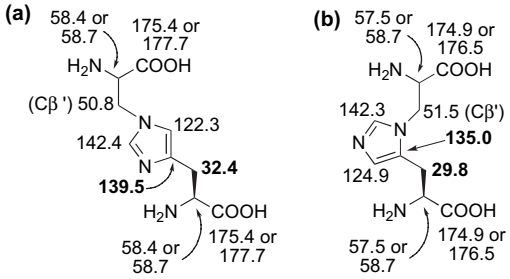


Figure 3. ^{13}C NMR data of (a) τ-HAL and (b) π-HAL (D_2O, pD 7, 22.5 MHz).

The ^{13}C NMR chemical shifts reported by Sass and Marsh[3,4] are summarized in Figure 3. In both compounds, there is a notable downfield shift for Cβ′ (relative to Cβ in Ala) since it is directly attached to nitrogen. The resonances that vary most between the two regioisomers (bold, Fig. 3) are Cβ (of the His residue) and C4 of the imidazole.

Boschin et al. have been the only group to address the fact that each regioisomer is predicted to be a mixture of diastereomers, varying in configuration at Cα of the 'Ala' residue.[6] Even at 600 MHz, they could not see any significant chemical shift differences for the two diastereomers in the ^{1}H NMR spectrum. Unfortunately, they do not reveal the solvent (or pH) in which their spectra were recorded, so it is not possible to draw comparisons. Nevertheless, some peaks were resolved in the ^{13}C NMR spectrum and those are highlighted in Figure 4.

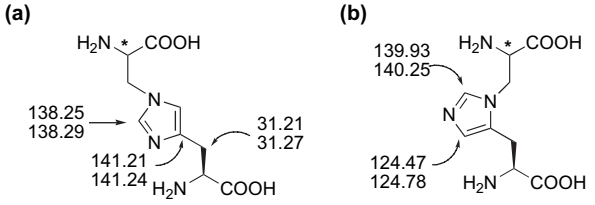
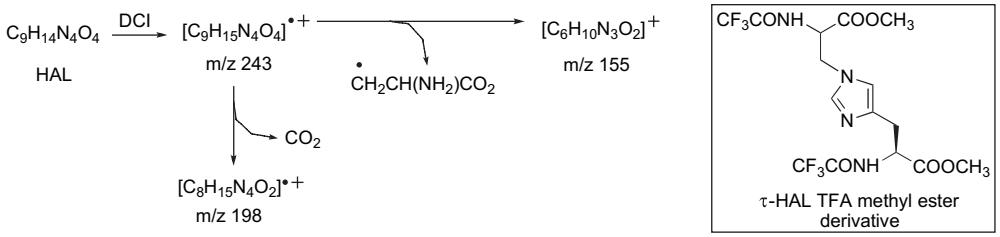


Figure 4. ^{13}C NMR signals that are resolved for the two diastereomers according to Boschin et al.: (a) the τ-isomer and (b) the π-isomer;[6] *=mixture of *R* and *S*.

Desorption chemical ionization (DCI) mass spectrometric data for τ-HAL and its trifluoroacetyl methyl ester derivative were reported by Fujimoto et al.,[2] and also by Sass and Marsh.[3,4] The major fragmentations are illustrated in Scheme 1. Both isomers behaved similarly.

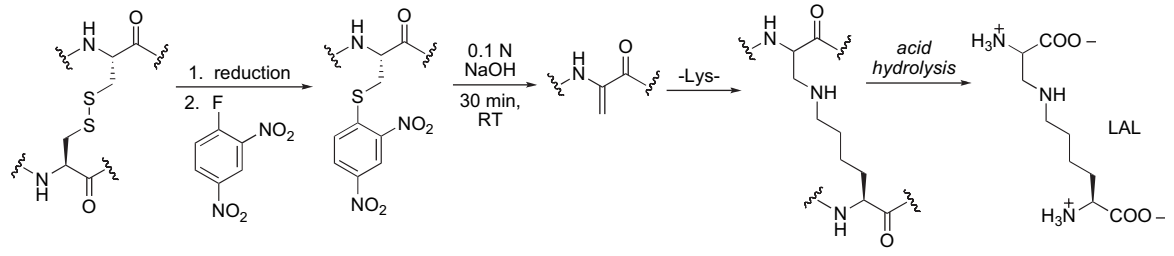


Scheme 1. DCI fragmentation of HAL.

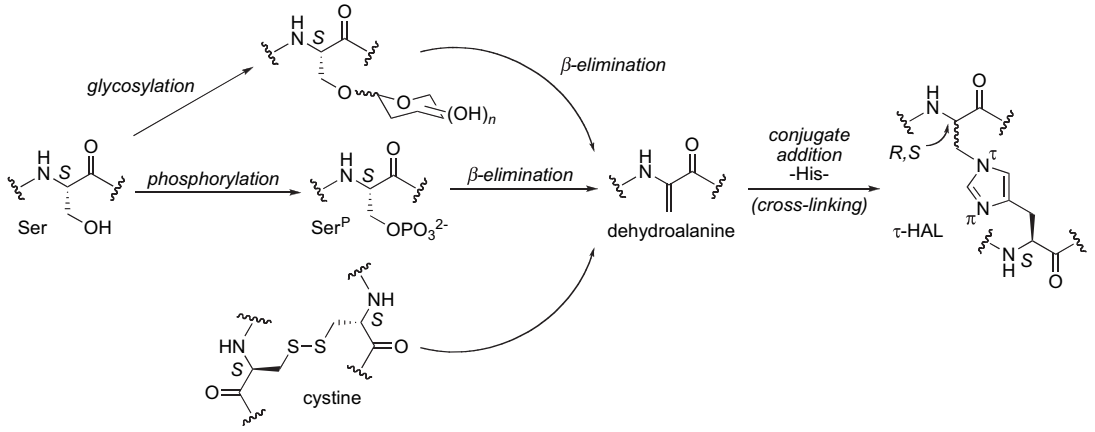
2. Mechanism of formation

A related crosslinking amino acid, lysinoalanine (LAL), was first identified in 1964.[7] Bovine pancreatic ribonuclease was reduced and the resulting thiols were derivatized as dinitrophenyl thioethers (Scheme 2). On treatment with alkali, the eight derivatized Cys residues formed dehydroalanine. Of these, three underwent conjugate addition to form lysinoalanine. This was supported by the observation that only 6.5 mol of Lys (of the eight in the original protein) was evident in the amino acid analysis.

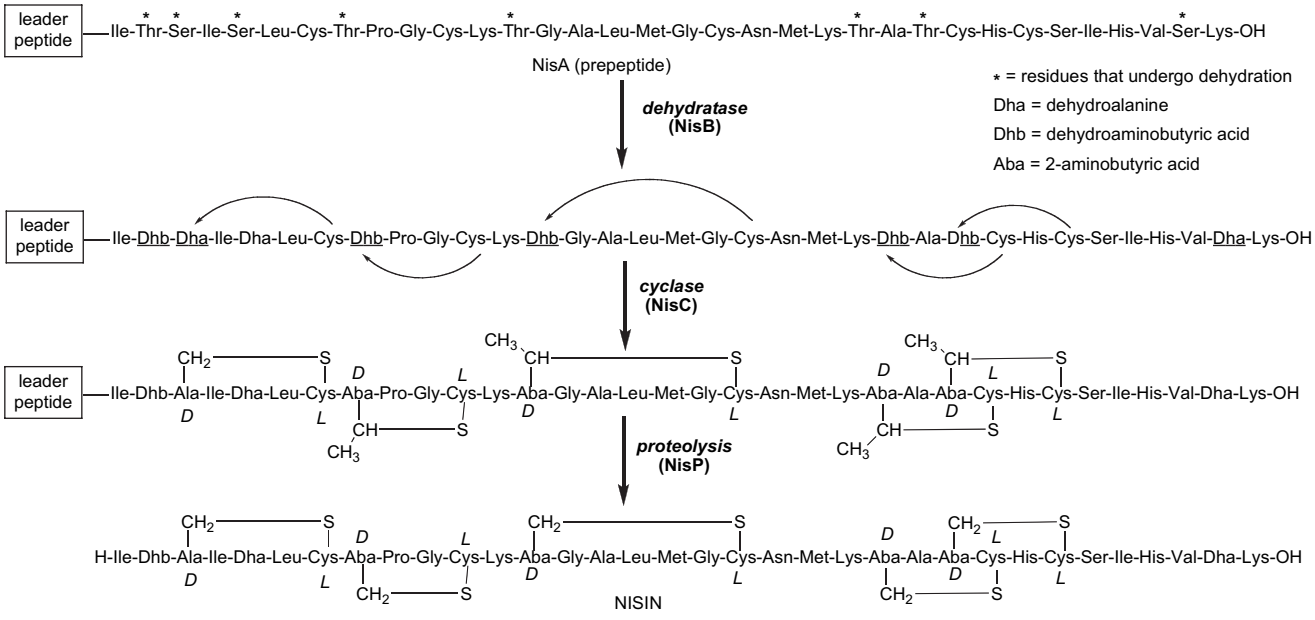
The mechanism for the formation of HAL has been presumed to proceed by analogy (Scheme 3). High levels of phosphoserine in many of the proteins that contain HAL (vide infra) suggest that β-elimination of phosphate is the



Scheme 2. Generation of LAL from RNase.



Scheme 3. Biogenesis of HAL.



Scheme 4. Steps leading to the production of nisin.

likely source of dehydroalanine. Other species that might give rise to dehydroalanine via β-elimination are cystine and serine *O*-glycosides. Conjugate addition of one of the imidazole nitrogen atoms of histidine to the dehydroalanine forms HAL.

The formation of HAL has been presumed to be a base-catalyzed, non-enzymatic process. However, there are enzymes known to dehydrate Ser and Thr to dehydroalanine (Dha) and dehydroaminobutyric (Dhb) acid, respectively. The enzymes associated with the biosynthesis of the lantibiotics have been studied in some detail.[8] In the synthesis of nisin, eight β-hydroxyamino acids are dehydrated enzymatically (Scheme 4). A second enzyme then catalyzes the stereospecific and regiospecific additions of five Cys side chain thiols to dehydroamino acids. The leader peptide, which may be important for recognition by the enzymes involved in this series of reactions, is then cleaved to give nisin, containing five cyclic thioethers.

It is entirely possible that enzymes may be involved in the generation of HAL in some instances. This is likely in the lens crystallins where the pH is neutral and the precursor amino acid is serine itself (cf., phosphoserine). The theonellamides are another case where enzymatic intervention is likely.

3. Food products

Heat and alkali treatments are widely used in the processing of protein-containing foods.[9] For instance, in the preparation of soy protein concentrates, the beans are extracted with aqueous alkali and then the protein precipitated at its isoelectric point. Similar treatments are used in recovering proteins from grains, oilseeds, and milk. Under the highly alkaline conditions, where the side chain of lysine is present as its free amine, significant levels of lysinoalanine (LAL) are formed.

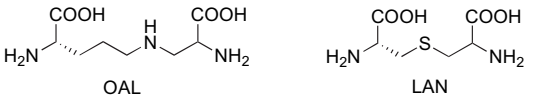
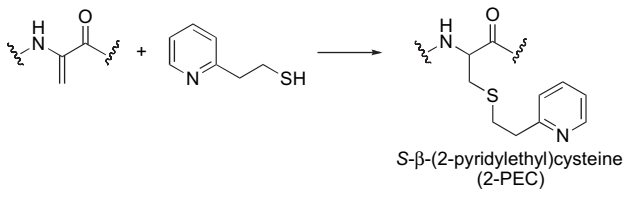


Figure 5. Structures of other crosslinking amino acids.



Scheme 5. Derivatization of dehydroalanine.

Finley and Friedman postulated that dehydroalanine might react with other amino acid side chain nucleophiles, including His, Orn, and Cys to give HAL, ornithinoalanine (OAL) and lanthionine (LAN), respectively (Fig. 5).[1] Lanthionine is the bis-amino acid derived from dehydroalanine and cysteine in the lantibiotics (Scheme 4). They synthesized these putative crosslinking amino acids and demonstrated good agreement in HPLC retention times with compounds in a soy protein hydrolyzate.

Masri and Friedman developed a method for the indirect detection of dehydroalanine in proteins.[10] Formation of a cysteine derivative, via conjugate addition (Scheme 5), provides an amino acid that is stable and amenable to amino acid analysis.

Fujimoto reported the formation of HAL in various proteins upon heating at neutral pH (Table 2).[11] The HAL content was assayed by acid hydrolysis of the protein followed by HPLC (two different solvent systems) and by two-dimensional TLC on cellulose. In a bovine serum albumin (BSA) sample, four residues of HAL were present in each protein molecule after heating at 110 °C for 96 h. There was also some evidence that the rate of HAL formation was temperature dependent. Fujimoto suggested that HAL crosslinks are formed during cooking, both in home and in industry.

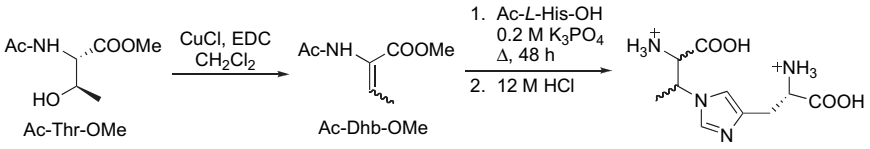
Table 2. Formation of HAL in heated proteins[a,11]

Protein	Bovine serum albumin	Casein	Ovalbumin	Human γ-globulin	Porcine pepsin	Bovine tendon collagen
HAL formed (nmol/mg)	39.0	15.3	8.3	17.7	7.0	0.7

[a] Experimental conditions: 10 mg of protein sample was heated at 110 °C for 24 h in 1 mL of potassium phosphate buffer (0.1 M, pH 6.8).

Henle et al. performed amino acid analysis on the hydrolyzates of skim milk.[5] The major milk protein is casein, a phosphoprotein. Their analysis involved ion exchange chromatography of the ninhydrin-derivatized hydrolyzate (and standards) with detection at 440 and 570 nm. They were able to isolate both regioisomers (τ/π ratio 8:1) from a severely heat-treated milk sample. The isolated compounds were then characterized by NMR. They demonstrated a linear relationship between τ-HAL concentration and the length of time the milk samples were incubated at 100 °C, presumably at neutral pH. They also identified HAL in a variety of milk products, at levels ranging from 50 to 1800 mg/kg of protein.

In a subsequent paper, Walter et al. prepared histidinomethylalanine (HMeAL, Scheme 6). Despite the availability of this synthetic sample, they were unable to detect this analog (derived from Thr instead of Ser) in the acid hydrolyzates



Scheme 6. Synthesis of histidinomethylalanine (HMeAL).

of a variety of milk products. The detection limits were 20–30 pmol.[12]

Fujii and Koboki reported the occurrence of HAL in lime-processed Indian bone gelatin, presumably of bovine origin.[13] Commercial gelatin, for use in the food industry, is produced in a variety of ways, including highly alkaline conditions (CaO, viz. lime-processing). Gelatin itself is derived from denatured collagen, but other non-collagenous proteins are known to be present in small amounts and these are responsible for turbidity. These proteins were separated by DEAE-cellulose chromatography and pepsin or trypsin digestion. They were found to contain high levels of glycoprotein, rich in Asp, Glu, and Leu. The pepsin precipitate was determined to contain HAL (8 residues per 1000) and LAL (27 residues per 1000). The major monosaccharide appeared to be galactosamine.

The effect of protein crosslinks on the nutritional value of food is largely unknown. Certainly, covalent crosslinks like HAL and LAL increase the stability toward proteolytic enzymes, thereby making them more difficult to digest. They also lead to a reduction in histidine and lysine, respectively, which may have a nutritional cost.

Toxicity studies in rats have shown that feeding high levels of alkali-treated protein leads to nephrocytomegaly, a disorder of kidney cells.[14] This observation has been ascribed to lysinoalanine. Two reports have appeared that have attempted to correlate HAL levels with age.[15]

Fujimoto reported that levels of HAL in urine range from 1.330±27 nmol/mg creatine in newborn babies, to 0.77±0.23 nmol/mg creatine in children to 0.89±0.33 nmol/mg creatine in adults.[16] He proposed that the urinary HAL was likely derived from proteoglycans.

4. Connective tissues

The aorta is the largest artery in the body. The ascending aorta rises from the left ventricle of the heart, arches, and then descends and branches into the thoracic aorta (above the diaphragm) and the abdominal aorta (below the diaphragm). The aorta is elastic and the ability to expand is important for blood pressure. As we age, this elasticity decreases. One of the possible contributions to this is excessive crosslinking of the tissues.

Fujimoto investigated the levels of crosslinking amino acids in human aorta tissue.[17] Disease-free samples of aorta (nine subjects ranging in age from 20 to 80) were obtained at autopsies, defatted, and dried. Acid hydrolysis of the tissue was followed by chromatography, on a phosphocellulose column, to separate the 'mono' amino acids (eluted with 0.1 M HCl). Histidinoalanine and the other crosslinking amino acids (Fig. 6) were eluted with stronger acid.

The pool of crosslinked amino acids was TNT-derivatized by treatment with 2,4,6-trinitrophenylsulfonic acid at pH 8.[18] Presumably each primary amine in a crosslinking amino acid is derivatized (Fig. 7) and the extinction coefficients were taken into account to make comparisons. Okuyama and Satake have demonstrated that the imidazole in histidine does not react under these assay conditions.[18] Analysis of these derivatized amino acids was conducted by RP-HPLC with UV detection at 350 nm. Levels of desmosine and isodesmosine decreased slightly with age; pyridinoline increased slightly and histidinoalanine increased by nearly a factor of four over the age range. For a 66-year-old subject it was estimated that HAL constituted 14 residues per 1000.

In order to pinpoint the origin of the histidinoalanine, they treated the aortic tissue with elastase and collagenase prior to amino acid analysis. Both enzymes were necessary to solubilize all the histidinoalanine, indicating that HAL is associated with both collagen and elastin. It was proposed that HAL is an important crosslink between elastin and other proteins.

Hardening of the arteries involves calcification of the blood vessel walls [deposition of hydroxyapatite, $Ca_3(PO_4)_2$]. While the results above were derived from healthy aortic tissue, Fujimoto and Yu wanted to see how the distribution of these crosslinks was affected in the atherosclerotic disease state.[19] They compared tissue from different parts of the aorta of the same 64-year-old male and concluded that levels of HAL were highest near the atheroma (yellow, flaky material at the center of plaques) and atheroarterioschlerotic lesions. Comparison of samples from three other men with

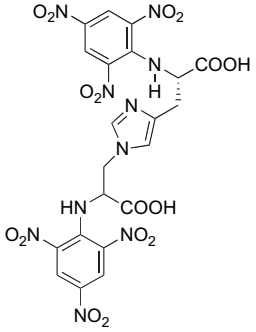


Figure 7. TNT derivative of τ-HAL.

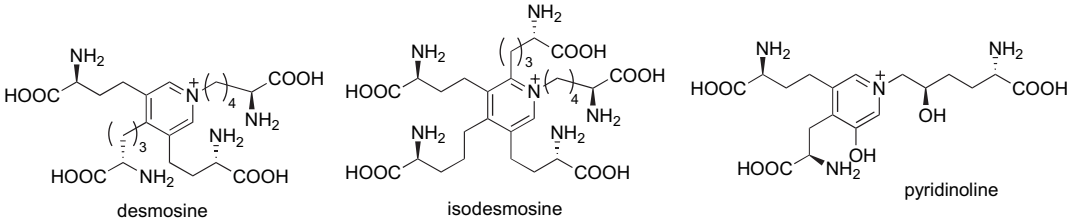


Figure 6. Other crosslinking amino acids found in aortic tissue.

varying degrees of atherosclerosis also indicated that HAL levels increased with the progression of the disease.

A much larger survey (aortic samples from 126 individuals) was conducted in 1987.[20] A linear correlation between age and HAL content was confirmed for both medial (middle) and intimal (innermost) layers of the aorta. The correlation coefficients were better for the pool of individuals with no evidence of atheroma or calcification.

Significant levels of HAL (>20 μmol/g in individuals over 60 years) were identified in costal cartilages;[21] analysis was conducted as described earlier. Costal cartilages connect the sternum to the ends of the ribs and their elasticity permits the chest to move during respiration. Treatment of the costal cartilage of a 61-year-old subject with collagenase released only a small amount of HAL, suggesting that it is present in non-collagenous proteins. Fujimoto proposed that the bulk of the HAL resides in proteoglycans that are abundant in cartilage.

This hypothesis was tested in collaboration with Roughley, by examining human articular cartilage (that surrounding joints).[22] Cartilage that appeared macroscopically normal was obtained at autopsy, diced and extracted with 4 M guanidinium chloride containing proteinase inhibitors. Proteoglycan subunits were recovered from the extract by cesium chloride density gradient centrifugation, collecting fractions with a density greater than 1.54 g/mL. Protein hydrolysis and analysis were then performed as above. Pertinent results are summarized in Figure 8.

Seyama et al. considered the changes in connective tissue composition in various diseases associated with the heart.[23] The tissues of various samples were extracted and partitioned into three fractions: glycoprotein, collagen, and elastin. There were at least five patients for each condition, but due to small amounts of tissue available during surgery, not all assays could be conducted for all patients. The data summarized in Table 3 thus represent a small survey.

Levels of glycoprotein (as gauged by the major component, sialic acid) and collagen (assessed by levels of hydroxyproline) did not show huge variations. The amount of elastin was decreased in disease (Table 3). Moreover, crosslinking is diminished, including a 3 to 5-fold decrease in the HAL content of the connective tissue overall. The authors proposed that reduced crosslinking makes the elastin more susceptible to degradation, including that by endogenous elastases.

The HAL content of mandibular bone in male rats (10 rats at each of the following ages: 1 month, 3 months, 12 months, and 24 months) was determined, by the analytical procedures described earlier. Analysis was conducted after removal of

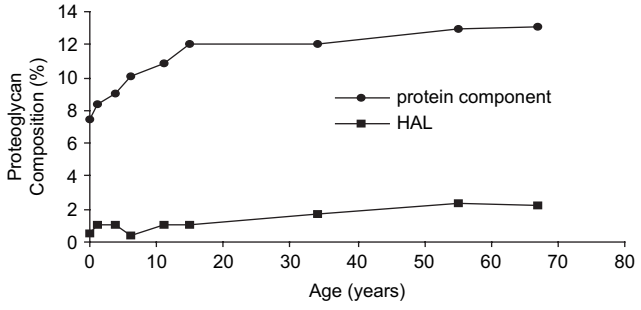
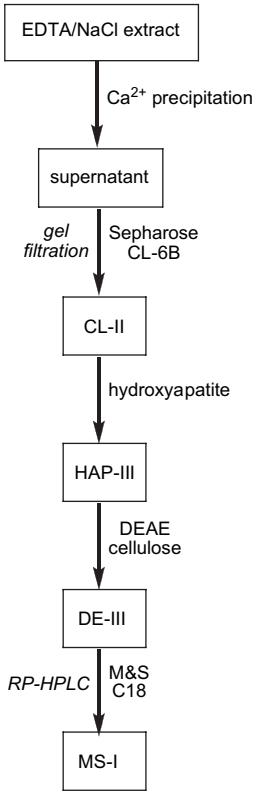


Figure 8. Variation in protein and HAL content of proteoglycan from articular cartilage.[21]



Scheme 7. Isolation of MS-I.

Table 3. Composition of connective tissue in various subjects[23]

Feature	Units	Aortic regurgitation[a] (control)	Marfan's syndrome[b]	Aortic aneurysm[c]	Dissecting aneurysm[d]
Sialic acid content of glycoprotein fraction	pmol/mg	34.1	39.3	24.5	30.8
Hyp content of collagen fraction	μg/mg	41.9	39.2	39.6	39.3
Purified elastin/elastin fraction	wt %	32.2	8.47	27.7	24.0
Isodesmosine content of elastin fraction	nmol/mg	13.8	3.49	3.38	3.83
Cys:Cys-Cys in elastin fraction	%	8.82	19.8	12.8	22.7
HAL in dry tissue	nmol/mg	14.6	3.17	4.85	3.73

[a] Defective valve between the left ventricle and aorta; left ventricle has to work harder to pump blood and becomes enlargened.
[b] Genetic disorder resulting in defective connective tissue; can manifest in the eyes, heart, skeletal system, skin, etc.
[c] Bulge in the aorta.
[d] Tear in the inner layer of the aorta, a rare condition, usually in 60–70 year old patients.

all the tissues, demineralization, removal of teeth, and defatting.[24] Levels were unchanged (~0.5 nmol/mg dry weight) during the first 3 months of life. This doubled to ~1 nmol/mg by 12 months and exceeded 2.5 nmol/mg by 24 months. There was good agreement between specimens within each age group. Shikata et al. suggested that HAL may serve as a marker for the senescence of mandibular bone.

Kuboki et al. isolated a 24K phosphoprotein from bovine bone that contained 1.2 mol of HAL per mole of protein.[25] The protein was isolated via several steps involving different types of chromatography (Scheme 7). At each step, the fraction richest in HAL was pursued further in terms of homogeneity. The amino acid composition of the phosphoprotein (MS-I) is depicted in Figure 9. The protein contains a high population of acidic residues (or their amides). There are a lot of serine and threonine and a significant amount of phosphoserine. There are also high levels of lysine and arginine. While the two crosslinking amino acids (HAL and LAL) have been combined and account for less than 1%, their presence is important. Interestingly, the ratio of HAL/LAL invariably seems to be 3:1.

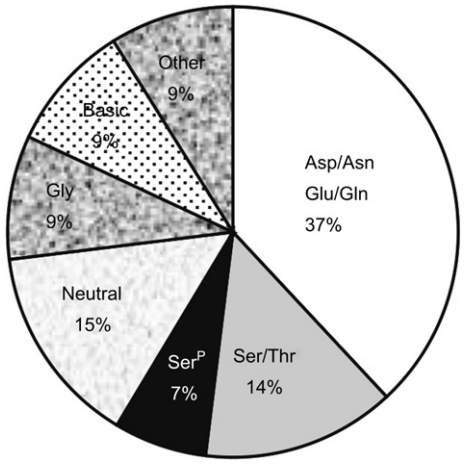


Figure 9. Amino acid composition of MS-I, the phosphoprotein isolated from bovine bone.[25] Others: Pro (6.8%), His (2%), and HAL and LAL (0.6%).

5. Dentin

The dentin matrix consists of about 90% type I collagen and a smaller amount of non-collagenous proteins (NCPs) including the phosphophoryns. Dimuzio and Veis described the biosynthesis of two phosphophoryns in the rat incisor, named α (first to elute from a DEAE-cellulose column) and β (second to elute).[26] These phosphophoryns have a similar amino acid composition to the bone phosphoproteins described above: more than 35% of the total residues is Asp and more than 40% is Ser or Ser^P. Dimuzio and Veis injected rats with [^3H]-Ser intracardially. There was evidence of radioactivity in the phosphophoryns within 15–30 min, reaching a maximum after 2 h that was sustained for 30 h. By comparison, collagen showed a steady accumulation of [^3H]-Ser and [^3H]-Gly. They proposed that the phosphophoryns play a key role in directing mineralization, given their localization and turnover at the mineralization front, in conjunction with their high negative charge and affinity for calcium ions.

In 1981, Fujimoto et al. first reported the presence of HAL in the acid hydrolyzate of human dentin and bone collagens.[2] They proposed that HAL, together with lysinoalanine, was responsible for the insoluble nature of the collagens in calcified tissues. However, a detailed investigation of HAL in bovine dentin by Kuboki et al. revealed that HAL distributed in the phosphoprotein fraction instead of the collagen fraction (Table 4).[27]

Kuboki's group separated the collagen and phosphoprotein fractions from the dentin of unerupted bovine incisors, by precipitation of the phosphoprotein with calcium ions. The phosphoprotein is divided into two fractions—that extractable into EDTA (free type) and that remained insoluble (bound type). Amino acid analysis was performed according to the Fujimoto protocol, with standards provided by Fujimoto.

There appears to be a complex formed between the phosphoprotein and collagen; the 'bound type' representing an 80:20 phosphoprotein/collagen composition. The free-type phosphoprotein is considered as a precursor to this complex, suggesting that covalent HAL and LAL crosslinks are important in the assembly of the complex. Kuboki et al. make the important point that there are no alkaline conditions during the isolation of their phosphoprotein samples. Thus, the crosslinks exist in the native protein and are not artifacts of the protein processing.

Ibaraki et al. also isolated two forms of phosphoryn from bovine incisor dentin and purified the major isoform (110 kDa) by DEAE-Sephacel and Sepharose CL-6B chromatography.[28] They prepared a 95% dephosphorylated version by treatment with alkaline phosphatase. They demonstrated that dentin phosphophoryn (DPP) is more resistant to proteolytic degradation than dephosphorylated DPP. They observed that DPP is sensitive to heat; presumably the degradation they observed at 100 °C is a result of dehydroalanine formation and subsequent events.

Cloos and Jensen performed a detailed study of the way in which the amino acid composition of dentin phosphoproteins changes with age (Figs. 10 and 11).[29] They looked at dentin from 14 intact, permanent, human teeth, covering an age range of more than 60 years; teeth from two cadavers were included in the study. Amino acid analysis showed that the collagen underwent little change vis-à-vis post-translational modifications and racemization. The phosphoproteins, on the other hand, showed five times as much

Table 4. Composition of bovine dentin[27]

Fraction	Yield of fraction (%)	HAL[a]	LAL[a]
Total insoluble dentin matrix	100	0.10	0.14
Collagen	99	<0.01	<0.01
Bound-type phosphoprotein	0.9	3.29	8.45
Free-type phosphoprotein	0.5	0.95	1.66

[a] Residues per 1000 amino acids.

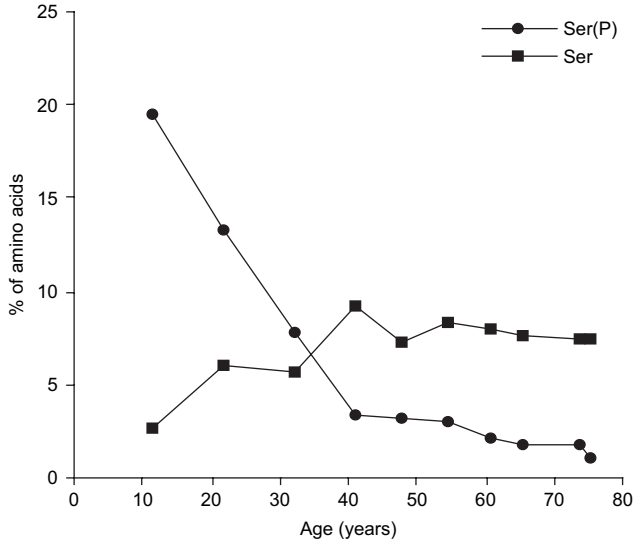


Figure 10. Variation in dentin Ser^P and Ser levels with age.[29]

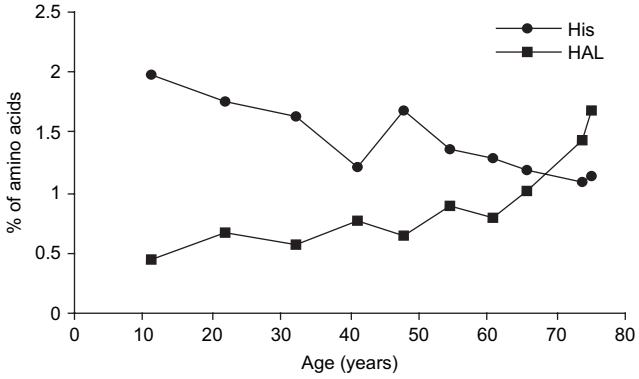


Figure 11. Variation in dentin His and τ-HAL levels with age.[29]

racemization of Asp and almost complete depletion of Ser^P over a lifespan.

Cloos and Jensen proposed that the analysis of Ser^P and/or HAL levels in dentin phosphoprotein might be useful in forensic applications, to estimate age at death. Teeth are good target for such analyses since they are generally preserved long after death.

6. Cataracts

The lens of the human eye contain ~38% protein (wet mass) of which the water-soluble crystallins account for more than 90%. The high concentration and degree of organization of α-, β-, and γ-crystallin are vital to maintaining the refractive index in the lens and thereby the focus aspect of vision.

With aging, a variety of post-translational modifications contribute to the aggregation and crosslinking of the crystallins. Srivastava et al. recently showed that Ser^{59} is converted to dehydroalanine in the α-crystallin in the multimer complexes of water-insoluble proteins.[30] These modifications lead to a reduction in water solubility. Lens crystallins do not undergo normal protein turnover, so modified proteins are retained throughout life. High levels of crosslinking can lead to lens opacity, which we know as a cataract. Cataract surgery involves replacement of the natural, damaged lens with an artificial intraocular lens (IOL).

In 1968, Pirie examined variation in color and solubility of lens proteins and thus helped to pinpoint the molecular nature of the aggregation.[31] She proposed a classification system based on the color of the lens after extraction: Group I (pale yellow) through to Group IV (dark brown/black, also knows as cataracta brunescens). Group I and Group II lenses were largely soluble in 6 M guanidinium hydrochloride after several hours at room temperature, implying that the disruption of hydrogen bonding was accomplishing solubility. Some materials remained insoluble in Group III lens; most of this could be solubilized by incubation with dithiothreitol. Thus, reduction of disulfide linkages was leading to 'resolubilization'. However, in Group III, and more so in Group IV, some insoluble materials persisted—suggesting the presence of crosslinks other than disulfide bridges.

In 1987, Kanayama detected HAL in human cataractous lens proteins.[32] Cataractous lenses from 14 eyes were obtained from patients aged 54–91. Four 'normal' lenses were obtained from recently diseased corpses. The lenses were analyzed for HAL as described above for other tissues (hydrolysis, TNT derivatization, and HPLC analysis). The mean values are illustrated in Figure 12, corroborating Pirie's observation that such crosslinks occur only in advanced stages of cataract growth.

More recently, Linetsky re-examined the occurrence of 'dehydroalanine crosslinks' in human lens (viz., LAL, HAL, and LAN).[33] They state that 'separation of 2,4,6-trinitrobenzene sulfonic acid-derivatized crosslinks by Kanayama's method resulted in the co-elution of all three crosslinks in one peak'. They go further, to suggest that Kanayama et al. effectively determined the total crosslinks in the lens, not HAL specifically. They were able to resolve the three bis-amino acids via their dansyl derivatives (e.g., Fig. 13). Their HPLC analysis utilized electrospray mass spectrometry as the mode of detection. Furthermore, LC–MS–MS was used to compare fragmentation patterns of authentic samples with the sample derived from the water-insoluble, cataractous lens proteins. Two peaks were observed for HAL, presumably the τ- and π-regioisomer. Lysinoalanine (LAL) was absent in normal lens proteins and only trace amounts

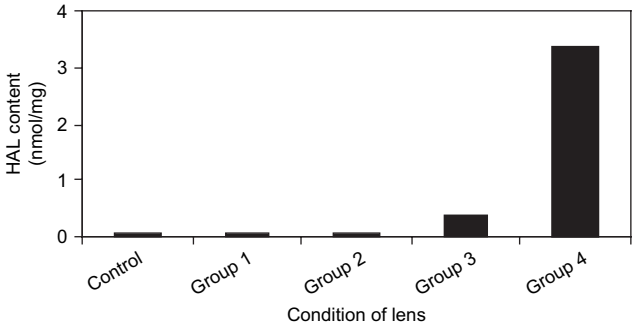


Figure 12. HAL content in human lens.

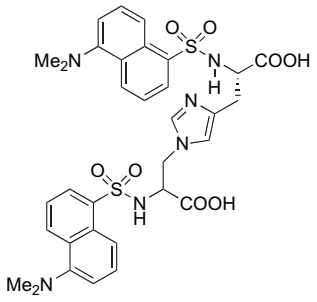


Figure 13. Dansylated τ-HAL.

were found in cataractous lens proteins (picomolar range). This is not surprising, given the neutral pH of the lens and that the pK_a of the ε-NH_3^+ group of lysine is 10.53. Only a small fraction of the ε-amino group is present as a free, nucleophilic amine under these physiological conditions.

By comparison, the imidazole of histidine has a pK_a of 6.0 and significantly elevated levels of this crosslink are found in proteins derived from cataractous tissue (Table 5).

Table 5. Levels of HAL (nmoles/mg protein) in lens proteins[33]

Protein fraction	Normal	Cataractous
Water-soluble	0.26±0.06	1.68±0.75
Water-insoluble	0.26±0.06	0.73±0.17

Another consequence of the aging lens is an increase in fluorescence. It has been suggested that this arises from changes in the crystallins, specifically reaction of kynurenine (an oxidative degradation product of tryptophan) with His (Scheme 8) or Cys, and Lys, to a lesser extent, for the reasons outlined above.[34]

7. Phosphoproteins of bivalve mollusks

Phosphoprotein particles (PPPs) are discrete structures about 40 nm in diameter that occur in the extrapallalial fluid (EF), hemolymph, and innermost shell lamella (ISL, a single continuous layer forming the entire inner surface of the shell). PPPs do not occur in the bulk shell. These particles are isolated by cutting the adductor mussel (that joins the two shells), removing the animal, and draining the fluid from the tissue.[35] The fluid is centrifuged to remove cells and then filtered (5 μm, then 0.45 μm) to remove particulate matter. The PPPs are sedimented by centrifugation and washed with water.

Demineralization (removal of Ca^{2+}, Mg^{2+}, and inorganic phosphate ions) is achieved by treatment with EDTA.[36] This breaks ionic crosslinks between subunits to give material that is less compact and less viscous.

At this stage, the protein can be partitioned into a low molecular weight fraction (supernatant) and a high molecular weight fraction (pellets) by centrifugation.[37] The low molecular weight fraction accounts for about 8% of the protein. The subunits are non-identical, and have similar charge density but different molecular weights. Agarose gel electrophoresis reveals four major bands that appear to be monomer (139 kD), dimer, trimer, and tetramer. The monomer units are joined together by covalent crosslinks, shown to be histidinoalanine residues (Fig. 14).

Acid hydrolysis of the phosphoproteins and amino acid analysis have revealed high levels of aspartic acid, serine and/or threonine (largely phosphorylated), histidine, and histidinoalanine.[3,4,35] These amino acids account for 90–95% of the amino acid component of the PPPs. Ammonia analysis indicates that ~10% of the Asp residues are in fact Asn residues. Other amino acids that occur in lesser amounts include lysine and glycine.[35]

Sass and Marsh were the first to identify the π-isomer of HAL.[3] They separated the monofunctional amino acids from the bis-amino acids on Dowex 50W-X8 (200–400 mesh, H^+ form). The bis-amino acids came out later, on increasing the acidity of the eluant. The two regioisomers were then separated on the same resin, eluting with a sodium citrate buffer. The ratio of τ-HAL to π-HAL was 3:1.

Sass and Marsh conducted a survey of the occurrence of PPPs in various bivalves including Heterodonts (clams,

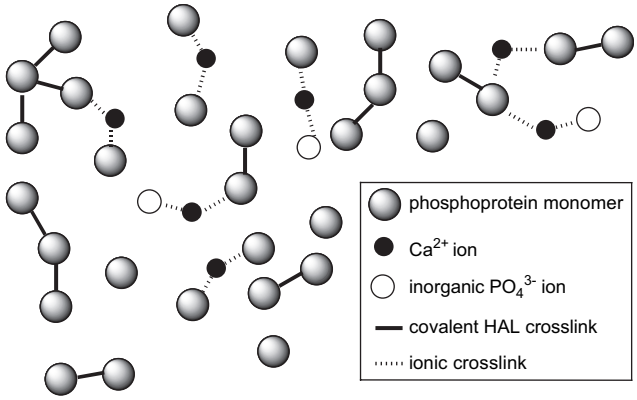
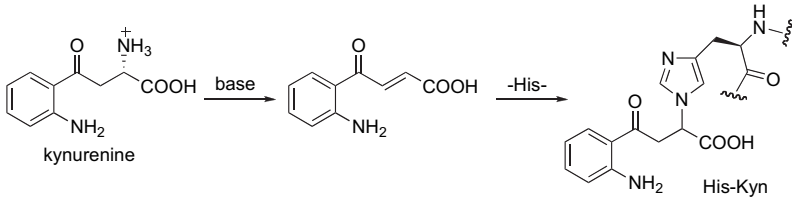


Figure 14. Schematic representation of the structure of the low molecular weight fraction of phosphoprotein particles.



Scheme 8. Formation of histidine–kynurenine conjugate.

cockles) and Pterimorphs (oysters, scallops, and mussels).[38] They concluded that all Heterodont species produced PPPs but the particle concentration varied, even between individuals of the same species. They did not find PPPs in any other subclass of Bivalvia.

It is also interesting to note that 'the phosphoprotein particles of all species are glycoproteins'.[35] Glucosamine, galactosamine, glucose, mannose, and fucose were identified in the hydrolyzates of PPPs from *Rangia cuneata*, accounting for 10% of the glycoprotein by weight. No attempt has been made to pinpoint the sites or patterns of glycosylation.

Waite and Qin described the isolation and characterization of a polyphosphoprotein (Mefp5) from the adhesive pads of *Mytilus edulis* (edible blue mussel).[39] This 74-residue protein has a mass of 9500 Da and the major amino acids are Gly (15 residues, 20.7%), DOPA (19 residues, 25.5%), and Lys (15 residues, 19.5%). There are lesser amounts of Ser (eight residues of which seven are typically phosphorylated, 10.8%) and His (five residues, 6.5%). It is possible that HAL crosslinks form in this protein.

In summary, histidinoalanine plays an important role in mineral binding in these mollusks, since its crosslinking role influences the quaternary structure of the PPPs. It is not clear how the PPPs (containing phosphate anions) participate in the deposition of the shell that consists of calcium *carbonate*.

8. Theonellamides

The theonellamides (Fig. 15 and Table 6) are a family of bicyclic peptides, characterized by a bridging

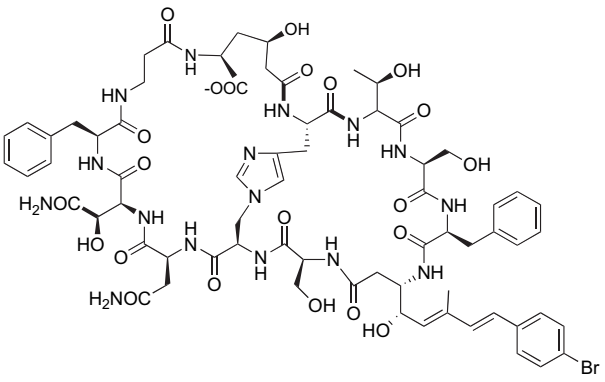


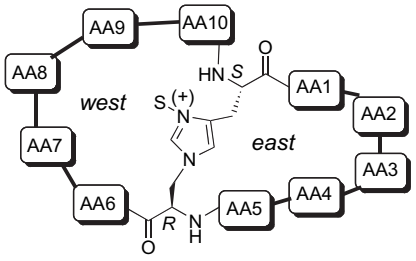
Figure 15. Theonellamide F.

τ-histidinoalanine residue. Some members of the family are further embellished by a monosaccharide (designated as S in Table 6) that is covalently linked to the π-nitrogen of the τ-HAL residue in the form of a glycosylimidazolium ion.

These complex natural products were isolated from marine sponges of the genus *Theonella* and characterized by the groups of Fusetani[40] and Faulkner[41] between 1989 and 1998. At the time of isolation, the compounds were reported to inhibit the growth of prototypical fungi (species of *Candida*, *Trichophyton*, and *Aspergillus*) and Fusetani's group reported cytotoxicity against P388 murine leukemia, with IC_{50} values ranging from 0.9 (theonellamide E) to 5.0 (theonellamide A) μg/mL.

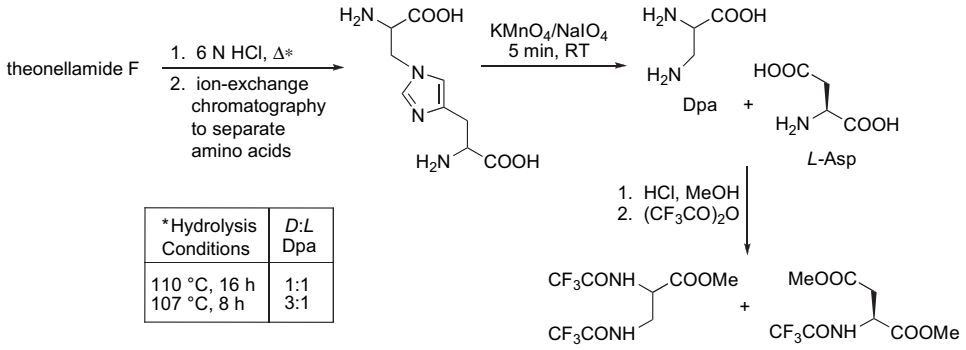
Matsunaga et al. determined the absolute stereochemistry of the τ-HAL residue from theonellamide F by degradation (Scheme 9).[40a] Oxidative cleavage of the imidazole ring yields 1 equiv each of 2,3-diaminopropionic acid (Dpa)

Table 6. The theonellamides



Amino acid	Theonellamide congener							
	A	B	C	D	E	F	Theonegramide	Theopalauamide
AA1	*allo*-Threonine							
AA2	Serine							
AA3	Phenylalanine							
AA4	Apoa	Apoa	Aboa	Aboa	Aboa	Aboa	Apoa	Apoa
AA5	Serine							
AA6	Asparagine							
AA7	(2*S*,3*R*)-3-Hydroxyasparagine							
AA8	BMPA	BMPA	Phe	BPA	BPA	BPA	BMPA	BMPA
AA9	Iser	β-Ala	β -Ala	β-Ala	β-Ala	β-Ala	Iser	Iser
AA10	Ahad	Ahad	Ahad	Ahad	Ahad	Ahad	AAA	AAA
S (sugar)	β-D-Gal	—	—	β-L-Ara	β-D-Gal	—	β-*D*-Ara	β-D-Gal

Abbreviations: AAA=α-aminoadipic acid; Aboa=(5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-*p*-bromophenyl-5,7-octadienoic acid; Ahad=α-amino-γ-hydroxyadipic acid; Apoa=(5*E*,7*E*)-3-amino-4-hydroxy-6-methyl-8-phenyl-5,7-octadienoic acid; Ara=arabinose; BMPA=β-methyl-*p*-bromophenylalanine; BPA=*p*-bromophenylalanine; Gal=galactose; Iser=isoserine.



Scheme 9. Degradation of theonellamide F by Matsunaga et al.

and L-aspartic acid (Asp). These were derivatized and analyzed by chiral GC–MS. The isolation of L-Asp was an indication of L-His in the natural product. The Dpa was less clear-cut; it appeared that epimerization was taking place during the acid hydrolysis. This was corroborated by the fact that different results were obtained when hydrolysis was conducted under milder conditions. The major enantiomer of Dpa was D, signaling that this was the natural configuration in the intact natural product.

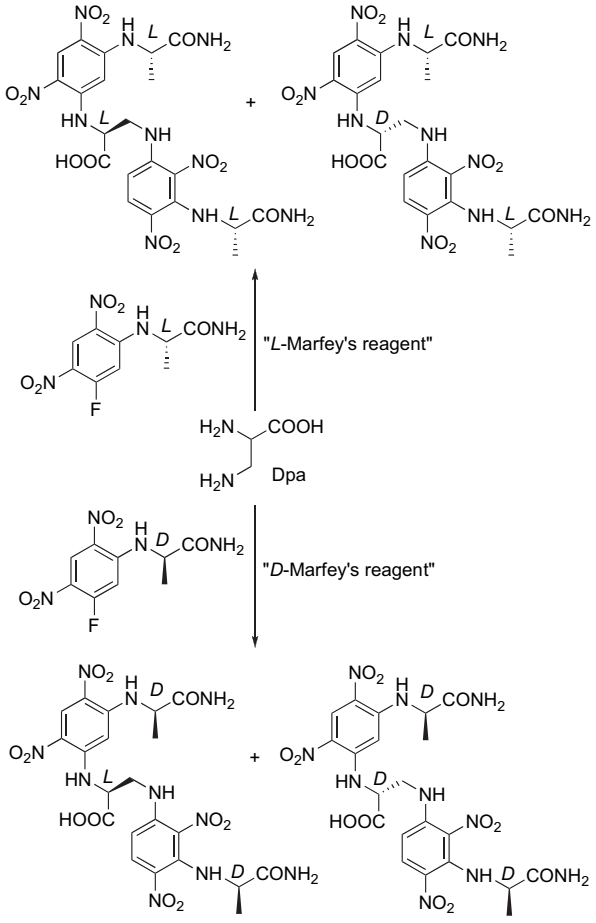
In their subsequent paper, where they revealed the structures of theonellamides A–E, Matsunaga and Fusetani confirmed this stereochemical assignment by derivatization of the diaminopropionic acid from the mild acid hydrolyzate with both enantiomers of Marfey's reagent (Scheme 10).[40b] Presumably, both primary amines of Dpr react in the nucleophilic aromatic substitution reaction to give the bis-Marfey adducts depicted. All four compounds were chromatographically resolved and confirmed the previous assignment.

Faulkner et al. reached the same conclusions about the absolute configuration of the two stereocenters in τ-HAL in theonegramide and theopalauamide, although their analysis was slightly different (Scheme 11). They degraded the imidazole via ozonolysis and derivatized the amines as pentafluoropropionamides. They did not detect any L-aminopropionic acid in their analysis, implying that their degradation and derivatization did not lead to epimerization of this residue.

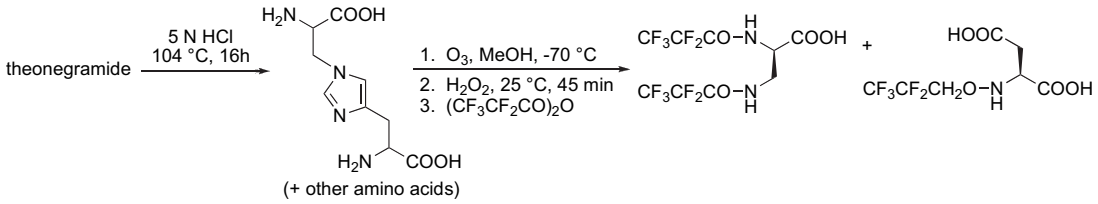
Compounds isolated from sponges often display structural similarity to microbial metabolites and so it is presumed that they arise from symbiotic microorganisms living within the sessile marine organism. The theonellamides are a rare case for which evidence has been provided for this hypothesis. In 1996, Bewley et al. reported the identification of four distinct cell types present in *Theonella swinhoei* from Palau;[42] they demonstrated that theopalauamide occurred only in the filamentous heterotrophic bacteria. Subsequent studies showed that the bacteria belong to the δ-subdivision of proteobacteria and the taxonomic status '*Candidatus* Entotheonella palauensis' was proposed.[43]

Watabe et al. have studied the behavior of theonellamides A and F in a variety of cell types. They reported that theonellamide F induced vacuole formation in 3Y1 rat embryonic fibroblasts.[44] The theonellamide F-induced vacuoles contain an ATPase and thus maintain a slightly acidic pH. The formation of vacuoles (fluid-filled compartments in the cytoplasm) is a well-known marker of microbial activity. The theonellamides are the first low molecular weight compounds demonstrated to induce extraordinarily large vacuoles. Surprisingly, they do not kill the cells, suggesting the potential as good molecular probes to investigate intracellular membrane structures.[45]

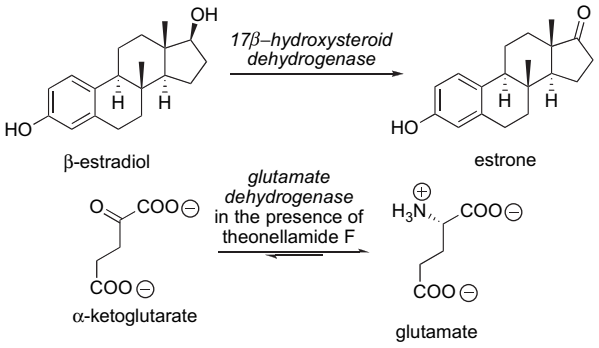
Wada et al. have established that theonellamides A and F bind to two enzymes from rabbit liver: 17β-hydroxysteroid dehydrogenase IV and glutamate dehydrogenase (Scheme 12).[46] Theonellamide F activates the conversion of α-ketoglutarate to glutamate, but has no effect on the reverse reaction.



Scheme 10. Marfey analysis of Dpa derived from τ-HAL.



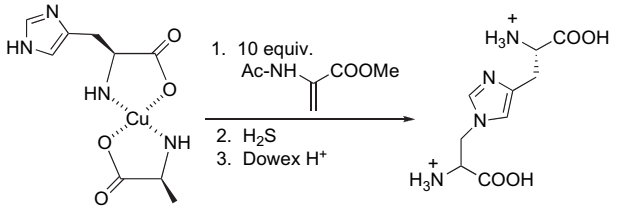
Scheme 11. Determination of τ-HAL stereochemistry by Faulkner et al.



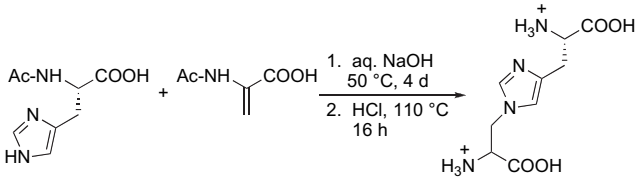
Scheme 12. Modulation of enzyme activity.

9. Synthetic efforts

As noted in Section 1, Finley and Friedman were the first to propose the existence of HAL. In their 1977 paper, they prepared crosslinking amino acids from methyl 2-acetylaminoacrylate according to Scheme 13.[1] The nucleophilic side chain functionality (τ-NH in the case of His) was assumed to undergo a conjugate addition. The resulting bis-amino acid was decomplexed from copper and purified by ion exchange chromatography. No characterization data were provided, other than HPLC traces and retention times.



Scheme 13. Finley and Friedman's approach to HAL.[1]



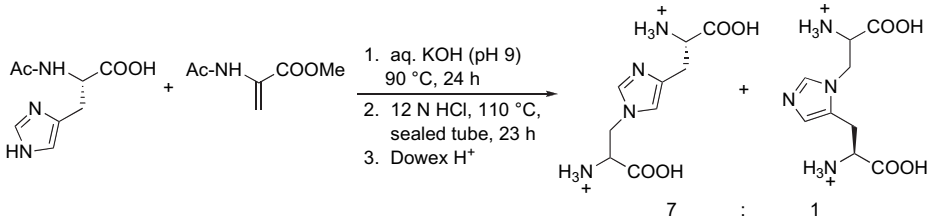
Scheme 14. Fujimoto's synthesis of HAL.[2]

Likewise, Fujimoto et al. prepared HAL in order to provide an authentic sample for their amino acid analysis (Scheme 14).[2] They used the free acid rather than the methyl ester of 2-acetylaminoacrylic acid. The HAL produced was purified by phosphocellulose chromatography and proton NMR data were reported (vide supra). They observed an 'impurity' in the NMR of their synthetic HAL and they attributed this to a small amount of the π-isomer.

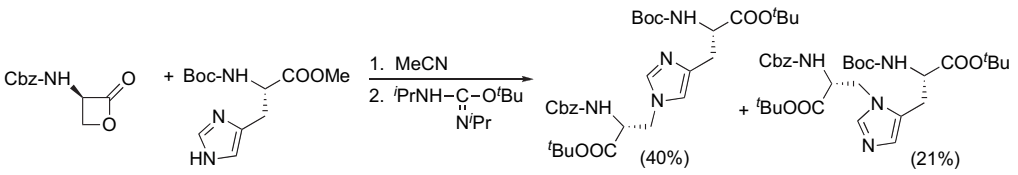
The first synthesis with full experimental details was published by Henle et al. in 1993. They used equimolar amounts of the two reactants to produce a mixture of the τ- and π-isomer (Scheme 15). The regioisomers were separated on Dowex 50W-X8 (H^+ form) eluting with 0.35 N sodium citrate (pH 5.35) with subsequent desalting. The two compounds were characterized by proton NMR.

Boschin et al. conducted their synthesis via the same general procedure as Henle.[6] However, they reported that lower temperatures and longer reaction times for the conjugate addition step were beneficial to the yield of the reaction. After 10 days at room temperature (0.3 N NaOH) they isolated a 5:2 mixture of τ- and π-HAL in 94% overall yield. They claim to have isolated and separated these regioisomers by flash chromatography on silica gel, eluting with 5:4.5:1:0.5 $MeOH/CH_2Cl_2/H_2O/NH_3$.

Tohdo et al. published several reports toward the total synthesis of theonellamide F.[47] As such they required a stereoselective synthesis of HAL, i.e., not a mixture of diastereomers, as is inevitable from the conjugate addition reactions described above. They employed a β-lactone derived from D-serine and thus controlled the configuration at both stereocenters. They still obtained a mixture of



Scheme 15. Henle's synthesis of HAL.[5]



Scheme 16. Tohdo's synthesis of L-histidino-D-alanine.

regioisomers but the *tert*-butyl esters could be readily separated (Scheme 16).[47a,f]

10. Summary

Histidinoalanine is a protein crosslink that has been identified in a range of proteins from diverse sources. The linkage can be generated in vitro, as is the case in food processing. Under alkaline and/or thermal conditions that promote elimination of phosphate from phosphoproteins, dehydroalanine is generated. Under highly alkaline conditions, lysinoalanine is the major player. At lower pH, where lysine residues are protonated, histidinoalanine and lanthionine dominate the pool of crosslinks. While concern has been expressed about the nutritional impact of these crosslinks, there is little doubt that pasteurization of milk and the isolation of proteins by alkaline extraction are well established processes that appear overall to be beneficial to human health.

Some of the early literature erroneously reported the occurrence of HAL in collagen. This misconception arose because of the difficulty in separating the various proteins in tissue. More weight must necessarily be given to more recent reports where modern purification and analytical methods have been applied to ensure that results relate to relatively homogenous materials.

There can be little doubt that phosphoserine and histidine are the amino acid precursors to HAL in phosphoproteins. These proteins are negatively charged, due to their phosphorylation and high levels of acidic amino acids. The function of these proteins—in connective tissue, bone, dentin, and mollusks—seems to be related to calcium mineralization. Is HAL an artifact, or does it play a more important role? Certainly, in the case of the proteins derived from bivalve mollusks, HAL has been identified as the covalent crosslink between protein monomers. There appears to be a correlation between HAL levels in some human tissue with age and with heart disease.

The occurrence of HAL in advanced eye cataracts seems mechanistically and functionally different to the phosphoproteins. The precursor amino acid in the crystallins is unmodified serine and the medium of the lens is neutral in terms of pH. It seems likely that severe crosslinking and/or formation of large crystallin aggregates contribute to the insolubility and opacity of the lens.

The theonellamides are an architecturally fascinating family of molecules. While a number of biological activities have been reported, it is unclear what their role is in the bacteria/sponge symbiotic relationship. In terms of their biogenesis, it is tempting to suggest that the theonellamides are the histidine analogs of the lantibiotics.

It is hoped that this review has collected together the disparate reports of histidinoalanine over the past 30 years and tried to organize it in a way that presents the existing knowledge and suggests some common themes and purposes for this under-appreciated bis-amino acid.

References and notes

1. Finley, J. W.; Friedman, M. *Adv. Exp. Med. Biol.* **1977**, *86B*, 123–130.
2. Fujimoto, D.; Hirama, M.; Iwashita, T. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 1102–1106.
3. Sass, R. L.; Marsh, M. E. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 304–309.
4. Sass, R. L.; Marsh, M. E. *Methods Enzymol.* **1984**, *106*, 351–355.
5. Henle, T.; Walter, A. W.; Klostermeyer, H. *Z. Lebensm.-Unters. Forsch.* **1993**, *197*, 114–117; Data: τ-HAL (DMSO-d_6) δ 3.28 (d, J=12.3 Hz, 1H, Hβ^a), 3.32 (d, J=8.8 Hz, 1H, Hβ^b), 4.28 (m, 1H, Hα), 4.57 (m, 1H, Hα′), 4.79 (d, J=6.9 Hz, 1H, Hβ′1), 4.81 (d, J=4.8 Hz, 1H, Hβ′^{b}), 7.72 (d, J=9.5 Hz, 1H, H5), 8.72 (s, 3H, NH_3^+), 9.09 (s, 3H, NH_3^+), 9.25 (d, J=10.2 Hz, 1H, H2). π-HAL (DMSO-d_6) δ 3.28 (d, J=6.7 Hz, 1H, Hβ^a), 3.45 (d, J=5.0 Hz, 1H, Hβ^b), 4.39 (m, 1H, Hα), 4.65 (m, 1H, Hα′), 4.78 (d, J=5.6 Hz, 2H, Hβ′), 7.69 (d, J=12.0 Hz, 1H, H5), 8.86 (s, br, 6H, 2×NH_3^+), 9.30 (s, 1H, H2).
6. Boschin, G.; D'Agostina, A.; Arnoldi, A. *Food Chem.* **2002**, *78*, 325–331.
7. Patchornik, A.; Sokolovsky, M. *J. Am. Chem. Soc.* **1964**, *86*, 1860–1861.
8. (a) Sahl, H.-G.; Jack, R. W.; Bierbaum, G. *Eur. J. Biochem.* **1995**, *230*, 827–853; (b) Chatterjee, C.; Paul, M.; Xie, L.; van der Donk, W. A. *Chem. Rev.* **2005**, *105*, 633–683.
9. Friedman, M. *J. Agric. Food Chem.* **1999**, *47*, 1295–1319.
10. Masri, M. S.; Friedman, M. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 321–325.
11. Fujimoto, D. *Experientia* **1984**, *40*, 832–833.
12. Walter, A. W.; Henle, T.; Haeßner, R.; Klostermeyer, H. *Z. Lebensm.-Unters. Forsch.* **1994**, *199*, 243–247.
13. Fujii, T.; Kuboki, Y. *J. Soc. Photogr. Sci. Technol. Jpn.* **1985**, *48*, 359–366.
14. (a) Sternberg, M.; Kim, C. Y. *Adv. Exp. Med. Biol.* **1977**, *86B*, 73–84; (b) Feron, V. J.; van Beek, L.; Slump, P.; Beems, R. B.; Alder Nissen, J. *Biological Aspects of New Protein Foods*; Pergamon: Oxford, 1978; Vol. 44, pp 139–147.
15. (a) Wang, M.-F.; Lee, M.-I.; Wong, Y.-C. *Zhonghua Minguo Yingyang Xuehui Zazhi* **1995**, *20*, 229–238, article in Chinese, CAN 123:226955; (b) Nagamine, J.; Uezu, K.; Suzuki, I.; Asata, L.; Shinjo, S.; Yamamoto, S. *Hissu Aminosan Kenkyu* **1992**, *133*, 39–41, article in Japanese, CAN 121:81662.
16. Fujimoto, D. *J. Biochem.* **1986**, *100*, 809–811.
17. Fujimoto, D. *Biochem. Biophys. Res. Commun.* **1982**, *109*, 1264–1269.

18. Okuyama, T.; Satake, K. *J. Biochem.* **1960**, *47*, 454–466.
19. Fujimoto, D.; Yu, S. Y. *Biochem. Int.* **1984**, *8*, 553–560.
20. Sugiyama, T.; Fujimoto, D.; Arai, C.; Hasegawa, M. *Biomed. Res.* **1987**, *8*, 349–351.
21. Fujimoto, D. *Biochem. Int.* **1982**, *5*, 743–746.
22. Fujimoto, D.; Roughley, P. J. *Collagen Rel. Res.* **1984**, *4*, 395–398.
23. Seyama, Y.; Hayashi, M.; Usami, E.; Yamashita, S. *Amino Acids* **1992**, *3*, 287–292.
24. Shikata, H.; Hiramatsu, M.; Masumizu, T.; Fujimoto, D.; Utsumi, N. *Arch. Oral Biol.* **1985**, *30*, 451–453.
25. Kuboki, Y.; Takita, H.; Fujisawa, R.; Yamaguchi, H.; Yamada, H.; Tazaki, M.; Ohnuma, Y.; Mizuno, M.; Tsuzaki, M. *Calcif. Tissue Int.* **1991**, *48*, 196–203.
26. Dimuzio, M. T.; Veis, A. *J. Biol. Chem.* **1978**, *253*, 6845–6852.
27. Kuboki, Y.; Fujisawa, R.; Tsuzaki, M.; Liu, C. F.; Sasaki, S. *Calcif. Tissue Int.* **1984**, *36*, 126–128.
28. Ibaraki, K.; Shimokawa, H.; Sasaki, S. *Matrix* **1991**, *11*, 115–124.
29. Cloos, P. A. C.; Jensen, A. L. *Biogerontology* **2000**, *1*, 341–356.
30. Srivastava, O. P.; Kirk, M. C.; Srivastava, K. *J. Biol. Chem.* **2004**, *279*, 10901–10909.
31. Pirie, A. *Invest. Opthalmol.* **1968**, *7*, 634–650.
32. Kanayama, T.; Miyanaga, Y.; Horiuchi, K.; Fujimoto, D. *Exp. Eye Res.* **1987**, *44*, 165–169.
33. Linetsky, M.; Hill, J. M. W.; LeGrand, R. D.; Hu, F. *Exp. Eye Res.* **2004**, *79*, 499–512.
34. Vazquez, S.; Aquilina, J. A.; Jamie, J. F.; Sheil, M. M.; Truscott, R. J. W. *J. Biol. Chem.* **2002**, *277*, 4867–4873.
35. Marsh, M. E.; Sass, R. L. *J. Exp. Zoolog.* **1983**, *226*, 193–203.
36. Marsh, M. E.; Sass, R. L. *Biochemistry* **1984**, *23*, 1448–1456.
37. Marsh, M. E. *Biochemistry* **1986**, *25*, 2392–2396.
38. Marsh, M. E.; Sass, R. L. *J. Exp. Zoolog.* **1985**, *234*, 237–242.
39. Waite, J. H.; Qin, X. *Biochemistry* **2001**, *40*, 2887–2893.
40. (a) Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Wälchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582–2588; (b) Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1995**, *60*, 1177–1181.
41. (a) Bewley, C. A.; Faulkner, D. J. *J. Org. Chem.* **1994**, *59*, 4849–4852; (b) Bewley, C. A.; Faulkner, D. J. *J. Org. Chem.* **1995**, *60*, 2644; (c) Schmidt, E. W.; Bewley, C. A.; Faulkner, D. J. *J. Org. Chem.* **1998**, *63*, 1254–1258.
42. Bewley, C. A.; Holland, N. D.; Faulkner, D. J. *Experientia* **1996**, *52*, 716–722.
43. Schmidt, E. W.; Obraztsova, A. Y.; Davidson, S. K.; Faulkner, D. J.; Haygood, M. G. *Mar. Biol.* **2000**, *136*, 969–977.
44. Wada, S.; Matsunaga, S.; Fusetani, N.; Watabe, S. *Mar. Biotechnol.* **1999**, *1*, 337–341.
45. Wada, S.; Kantha, S. S.; Yamashita, T.; Matsunaga, S.; Fusetani, N.; Watabe, S. *Mar. Biotechnol.* **2002**, *4*, 571–582.
46. Wada, S.; Matsunaga, S.; Fusetani, N.; Watabe, S. *Mar. Biotechnol.* **2000**, *2*, 285–292.
47. (a) Tohdo, K.; Hamada, Y.; Shiori, T. *Pept. Chem.* **1992**, 7–12; (b) Tohdo, K.; Hamada, Y.; Shioiri, T. *Tetrahedron Lett.* **1992**, *33*, 2031–2034; (c) Tohdo, K.; Hamada, Y.; Shiori, T. *Pept. Chem.* **1992**, 149–151; (d) Tohdo, K.; Hamada, Y.; Shioiri, T. *Pept. Chem.* **1993**, 37–40; (e) Tohdo, K.; Hamada, Y.; Shioiri, T. *Synlett* **1994**, 105–106; (f) Tohdo, K.; Hamada, Y.; Shioiri, T. *Synlett* **1994**, 247–249; (g) Tohdo, K.; Hamada, Y.; Shioiri, T. *Synlett* **1994**, 250.

Biographical sketch

Carol Maree Taylor was born in Pukekohe, New Zealand in 1966. She received her B.Sc. in 1987 and her M.Sc. in 1988 from the University of Auckland, New Zealand. She received her Ph.D. in 1993 from the University of Pennsylvania (USA) under the direction of Professor Ralph Hirschmann and Professor Amos B. Smith, III. From 1993 to 1994, Carol was a Research Associate at Princeton University (NJ, USA) with Professor Dan Kahne. In 1995, she returned to New Zealand to the position of Lecturer at the University of Auckland where she was awarded continuation of appointment in 1998 and promoted to Senior Lecturer in 1999. In April 2000, she moved to Massey University in Palmerston North, New Zealand. In 2001, she received the RSC/NZIC Easterfield Award for original contributions by a New Zealand chemist under the age of 35 and was promoted to Associate Professor in 2003. In July 2006, she returned to USA as an Associate Professor at Louisiana State University in Baton Rouge. Her research program is primarily engaged with the chemical synthesis and physical characterization of novel peptide motifs that arise via post-translational modifications.

Weihua Wang was born in Laizhou, Shandong, China in 1969. She received her B.A. in Macromolecular Materials Science and Engineering from the Jiangsu Institute of Chemistry in 1992 and then worked as a research engineer with Yantai Plastics Corporation. She attended the Nanjing University of Technology, working on the preparation, characterization, and application of nano-silica/PMMA composites with Professor Yiqing Qian and was awarded her M.S. in 1999. Wang was then engaged as a Lecturer in the Department of Chemical Engineering at Jiangsu Polytechnic University. She began graduate school at Louisiana State University in 2001, investigating the organic chemistry and selective detection of biological thiols in the research group of Professor Robert M. Strongin. She received her Ph.D. in 2006 and is currently a postdoctoral researcher in Taylor group.