

Synthesis and Characterisation of 6, 10-diisopropyl-3-methyl-1,4,7-triazecane-2,5,8,9-tetraone/ NCA-VAV

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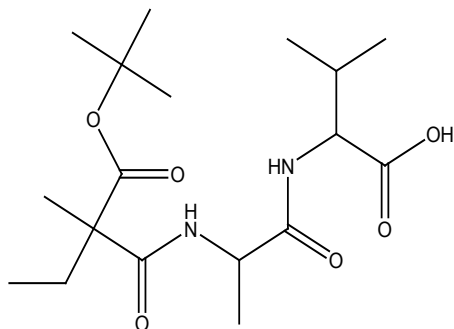
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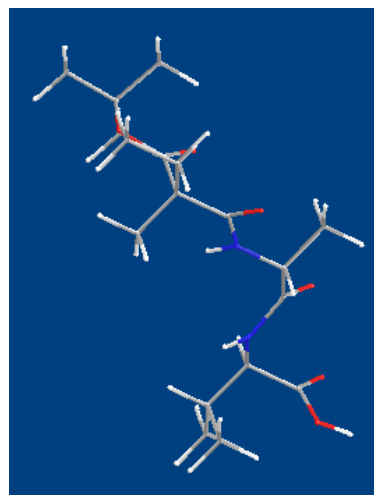
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Abstract NCAVAV= 2-(2-(2-(tert-butoxycarbonyl) methylbutanamido) propanamido)-3-methylbutanoic acid/ Boc-VAV-OH (Trimer) was synthesized by stepwise coupling of different amino acids. The 'Boc-VAV-OH' compound was, for the first time, cyclized to give a 6, 10-diisopropyl-3-methyl-1, 4, 7-triazecane-2,5,8,9-tetraone/ NCA-VAV monomer, using a Lewis acid. All compounds were characterized using UV, NMR, melting point, ESMS, TLC and FTIR



2-(2-(2-(tert-butoxycarbonyl)methylbutanamido)propanamido)-3-methylbutanoic acid



NCAVAV.

Keywords NCA-VAV, N-tert-butoxycarboanydrides

1. Introduction

Synthesis of NCAs' was initially carried out in the 1920s', using single amino acids and more recently by Deming et al. [1] [2] Thereafter interest in this area increased and the polylactides, polyglycolides and their co-polypeptides such as poly-ε-caprolactone-co-glycolic acid-co-serine (PCGS) [3] and poly-L-lactide-co-glycolide (PLGA)/polyethylene glycol (PEG) [4] were synthesized and are now used as substitutes for proteins. This incorporation of NCAs' into biomaterials [5]. Other applications for these homo- and

co-polypeptides include, tissue scaffolding, drug delivery and making of synthetic wool or silk substitutes. [6]

More recently, Deming et al [7] synthesized polypeptides using organometallic catalysts, under controlled conditions [8]. With this method, they synthesized polypeptides with molecular weights of up to 25 000 Da with low polydispersities. The group then went on to synthesize block co-polymers including poly-L-lactide-co-glycolide (PGLA) [9] and poly-ε-caprolactone-co-glycolic acid-co-serine (PCGS) [10] using the same catalyst. This was done by, introducing different monomers once the initial monomer was consumed.

Currently the three methods used to synthesize "NCA" monomers, are Leuch's, Fuchs Farthing and 'Silyazide'. Of these, the most commonly used methods are the Leuch's and Fuchs Farthing due to the ease of removal of bi-products.

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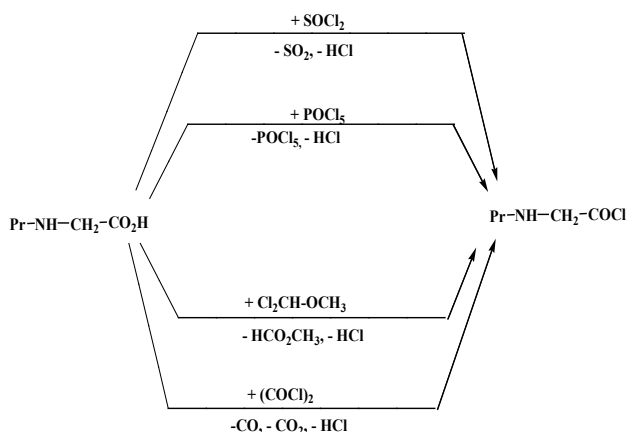
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The 'Fuch Farthing' method utilizes triethyl phosphate, phosgene or its derivatives (triphosgene), at low temperatures, in inert solvents such as dioxane and tetrahydrofuran (THF). [11] Triphosgene ($\text{CO}(\text{O}_2\text{CCl}_3)_2$) undergoes a nucleophilic attack at the carbonyl carbon, cleaving trichloromethoxy ($\text{Cl}_3\text{CO}-$) which then dissociates to a chloride anion and a molecule of phosgene, which then reacts immediately [12]. Triphosgene is often used as an alternative to phosgene, as it is safer, easier to handle and to purify [13]. However, triethyl phosphate can be used over shorter time periods and with few side reactions [14-16].

Fuchs Farthing is the best method for synthesizing NCAs' with N-substituted amino acids and amino acids with side groups like histamine (His), ornithine (Orn), lysine (Lys) and threonine (Thr). The reactive functional groups on these amines can be protected with a silane or benzyl (Ar) groups before NCA synthesis, to prevent side reactions that lead to difficult purifying procedures [17].

Leuch's method utilizes Lewis acids to convert the amino acids into acid chlorides e.g., N-ethoxycarbonyl and N-methoxycarbonyl amino acid Chlorides, [18-20] then to NCAs.' The Lewis acids used include acidic-bromides, such as phosphorus penta-bromide. They cyclize free amino acids more readily than the acid chlorides. [21] (See Scheme 1.1). Dichloromethyl methyl ether and oxalyl chloride were Lewis acids though other reagents such as Dimethylformamide (DMF) and tetrahydrofuran(THF) ether yield pure NCAs. However, in the cyclisation of α -diaminosuccinic acid, oxalyl chloride is preferred, as it does not interfere with the amino acid side groups due to its relatively low reactivity.



Scheme 1.1. Synthesis of NCA precursors from various reagents

However, slight variations to Leuch's method are necessary for cyclisation of special NCAs' from amino acids such as lysine, ornithine, arginine, glutamine and asparagine, to prevent acid-catalyzed side reactions such as hydrochloric or hydrobromic acid production. These amino

acids are initially silylated to reduce the side reactions. [22, 23]

In this article, the synthesis of cyclic monomers from their precursor peptides (dimers trimers and tetramers) will be described. The monomers will later then be used to polymerize stereo-specific polypeptides whose nature may offer some control over the polymers' properties. This can hopefully be used to control physiological behaviors of specific bio-systems. Synthesis of stereo-regular sequence polymers is a necessary step closer to preparing synthetic proteins with specific repeating sequences.

A standard method will thus be established (as showed in the scheme below) for synthesizing NCA monomers, from trimers in particular, characterizing their properties using melting point apparatus, product front Vs. Solvent front, FT-IR, ESMS and NMR.

- Where; V / Val = L-Valine; A / L-Ala = Alanine; OMe = Methyl ester protecting group for carboxylic acids,
- Boc = 4-*tert*-butoxycarbonyl for protecting amine functional group.

The sequences to be used include SIKVAV, which is the amine sequence of an active laminin found in most extra-cellular matrices of tissues. It was found to encourage cell adhesion, migration and vasculisation.

2. Results

Synthesis of 2-(2-(2-(*tert*-butoxycarbonyl) methylbutanamido) propanamido)-3-methylbutanoate/Boc-VAV-OMe

Yield: 72%, mp = 73.4°C, Chromatogram-DCM: MeOH, (20:1), R_f = 0.65; Boc-VAV-OMe, **MS** (EI: 4.15^o3 kV) m/z : δ 311.19 (2DIPEA+2Na⁺), 346.2 [14C₂₄H₅O₅N] +391.3 (M+15H⁺), 4.2.3 (M+Na⁺), 416.3 (M+Cl⁻+5H⁺); **FTIR (KBr discs)**: δ 2965 (s/str) CH₃, 2876 (s/str) CH₂, 2856 (s/str) CH₂, 2933 (s/str) CH₂, 1464 (m/def) CCH₃, 758 (s/ske)C(CH₃)₂, 1157 (s/ske) C(CH₃)₂, 1740 (s/str) C=O, 1669 (s/ str) C=O, 1542(s) C=O, 1521(s/str) C=O, 1236 (s/str) CN, 1377 (s/str) CN, 1216(s/str) C-O, **¹H NMR (400 MHz, CDCl₃)**: δ 0.83 (2H, dd(J =3.4, 3.0 Hz), β -(2CH)), 1.07 (2H, dd(J =6.3, 15.8, 6.8 Hz), β -(2CH)), 1.16 (6H, t(J =7.1 Hz), β -CH₃), 1.42 (9H, s, α (CH₃)₃), 2.21 (2H, m, α -(2CH)), 3.71 (3H, s(J =19.0 Hz), α -(O-CH₃)), 4.11 (1H, m, α -CH), 4.62 (1H, dt(J =6.5, 14.7 Hz), α -CH), 7.32 (1H, d(J =7.9 Hz), NH), 8.60 (1H, t(J =0.6 Hz) NH), 11.78 (1H, s, NH⁺), **¹³C NMR (100 MHz, CDCl₃)**: δ 16.51 (CH₃), 17.8 (CH₃), 19.91 (CH₃), 19.56 CH₃), 29.89 (3(CH₃)), 31.10 (CH), 31.17 (CH), 48.89 (CH), 49.1(CH), 52.41 (O-CH₃), 57.71 (CH₃), 60.19 (CH), 96.72 (C(CH₃)₃), 157.67 ν_{asy} (Boc C=O), 172.11 ν_{sy} (Amide C=O), 172.14 (Ala C=O) 173.04 ν_{asy} (Ester C=O).



Scheme 1.2. Synthesis of NCA-VAV/ 6, 10-diisopropyl-3-methyl-1,4,7-triazecane-2,5,8,9-tetraone

The methyl-protecting group on Boc VAV-OMe was removed as described by Joudaiye, J. et al. [24]

Synthesis of 2-(2-(2-(tert-butoxycarbonyl) methylbutanamido)propanamido)-3-methylbutanoic acid/Boc-VAV-OH

Yield: 82%, mp = 155.1°C, Chromatogram-DCM: MeOH (20:1) R_f = 0.22; **FTIR (KBr discs)**: δ 1496 (m/def) CH₂, 1391 (m/def) C(CH₃)₃, 1366 (m/def), 1418 (m/def) CCH₃, 1375 (m/def) CCH₃, 1709 (s/str) C=O, 1635 (m/str) C=O, 1506 (m/str) C=O, 1039 (m-w/str) CN, 1164 (m-w/str) CN, 1517 (s/str) COOH; **¹H NMR (300 MHz, DMSO-d₆)**: δ 0.74 (12H, m, α -(CH₃)₃, B-CH₃), 1.02 (3H, t(J =7.72 Hz), β -CH₃), 1.34 (6H, s, α -(CH₃)₃, 1.92 (2H, m, α -(2CH)), 2.70 (1H, quartet(J =7.0 Hz), CH), 3.83 (1H, dd(J =6.3, 1.9, 9.9 Hz), CH), 4.01 (1H, dd(J =5.3, 3.1, 5.5 Hz), α -CH), 4.37 (1H, t(J =7.3, 7.2 Hz), OH), 6.61 (1H, d(J =9.1 Hz), NH), 7.73 (1H, d(J =6.3 Hz), NH), 7.90 (1H, d(J =7.4 Hz), NH), **¹³C NMR (75 MHz, DMSO)**: δ 18.76 (CH₃), 18.79 (CH₃), 19.89 (CH₃), 28.09 (C(CH₃)₃), 30.09 (CH), 31.10 (CH), 46.18 (CH), 48.6 (CH), 58.66 (CH), 78.09 (C(CH₃)₃), 156.59 ν_{asy} (Boc C=O), 172.14 ν_{sy} (Amide C=O), 173.14 ν_{asy} (Amide C=O), 174.44 ν_{sy} (Val C=O). [25]

Cyclisation of 6, 10-diisopropyl-3-methyl-1,4,7-triazecan e-2,5,8,9-tetraone/ NCA/ VAV

Yield: 80%, m.p. = 120.8°C, Chromatogram-EthOAc: hex, (2:1), R_f = 0.4, NCA-VAV **MS**(EI -1.5^o7 kV) m/z : δ 327.2 (M+H⁺), 397.2 (M+3Na⁺), **FTIR (NaCl discs)**: δ 1435 (m/def) CH₃, 2924 (s/str) CH₂, 2853 (s/str) CH₂, 1587 (s/def) NH, 2490 (w/ str) NH, 2529 (w/str) C(CH₃)₂, 1174 (s/def) C(CH₃)₂, 1683 ν_{asy} (s/str) C=O, 1698 ν_{sy} (s/str) C=O, 1716 ν_{asy} (s/str) C=O, 1732 (s/str), 1748 (s/str), 1827 (s/str) O=C-O-C=O, 1790 (s/str) O=C-O-C=O., **¹H NMR (150 MHz, DMSO)**: δ 0.95-1.14 (12H, m, α (3CH₃), β -CH₃), 1.21 (3H, m, α CH₃), 3.61 (1H, m, α -CH), 3.71 (1H, t(J =4.2 Hz), α -CH), 4.25 (1H, q(J =4.8 Hz), β -CH), 8.07 (1H, s, NH), 7.71 (1H, d(J =8.9 Hz), NH), 7.25 (1H, s, NH), **¹³C NMR (150 MHz, DMSO)**: δ 22.6 (CH₃), 22.89 (CH₃), 24.51 (CH₃), 26.10 (CH), 29.20 (CH), 42.00(CH), 45.68 (CH), 51.21 (CH), 169.14 ν_{asy} (carbamate C=O), 172.11 (Val C=O), 173.13 ν_{sy} (Amide C=O), 174.27 ν_{asy} (Amide C=O)

Synthesis of 5-(((9H-fluoren-9-yl)methoxy)carbonyl)-2-(2-(6-(((9H-fluoren-9-yl)methoxy)carbonyl)-2-(tert-butoxycarbonyl)hexanamido)-4-methylpentanamido)-5-oxopentanoic acid/ NCA-KLK

Yield: 93%, mp 71.9°C, ACN/ Hex, (3:1), R_f 0.29: **FTIR (NaCl discs)**: δ 2940 (s/str) CH₃, 1151 (s/ske) C(CH₃)₂, 1666 (s/str) C=O, 1111 (m-w/ str/str) CN, 1084 (m-w/str) CN, 981 (s/def) Ar, 869 (s/def) Ar, 751(s/ def) Ar, **¹H NMR (600 MHz, DMSO)**: δ 1.04 [4H, s, β -(2CH₂)], 1.83 [2H, s, β -CH₂], 2.01 [4H, s, β -(2CH₂)], 2.19 [6H, d(J =3.6 Hz), β -(3CH₃)], 2.24 [5H, q(J =2.1 Hz), 2CH₂, α -CH], 2.40 [2H, s, β CH₂], 2.63 (1H, d(J =4.2 Hz), β -CH], 2.79 [1H, s, β -CH], 2.94 [1H, d, β -CH], 3.02 [1H, quartet, β -CH], 3.10 [1H, s, β -CH], 3.61 [1H, d(J =7.06 Hz), β -CH], 3.68 [2H, s, β -CH₂], 3.70 [2H,

quartet(J =3.4 Hz), CH₂], 4.23 [1H, t(J =7.0 Hz), α -CH], 6.06 [1H, s, β -CH₂], 7.64 [1H, t(J =0.7 Hz), NH], 7.78 [1H, s, NH], 7.93-8.00 [16H, s, fmoc], 8.17 [1H, s, NH].

3. Discussion

Amine functional groups of glycine and valine amino acids were protected with an *N*-tert butoxycarbonyl group, in basic conditions. The ESMS spectrum of 'NCA-VAV' is shown in figure 3, where 'M' denotes the 'compound.' The number of ions bonded to NCA VAV is shown together with the collective charge as salts. FTIR Spectrum of the amine protected amino acids showed deformation vibrations of 'Boc' protecting groups C(CH₃)₃, in the wave-number regions of (1,395-1,38) cm⁻¹. Spectral ¹HNMR data for Boc-V-OH have methyl functional groups 'C (CH₃)₃' of the 'Boc group' which is a characteristically tall peak in the spectrum between wave-numbers 1.3-1.5 ppm for all spectra

The carboxylic functional groups of the alanine and Valine groups were protected with an ester. IR spectrum gave information regarding the presence of twisting, bending and vibrating of the various functional groups in the compounds. The ester groups are found in the wave-number regions of 1,300-1,250 -110 cm⁻¹ and 1,150-1,110 cm⁻¹.

After coupling, there was an up-field shift in the amide functional groups to wave-number regions of 1220-1020 cm⁻¹ for the amines, a reliable indication of a successful coupling reaction. Protective esters on the carboxylic acid were not visible at wave-numbers ranging from 1264-1295 cm⁻¹.

¹H NMR shifts that correspond to the 'boc' group for amine and carboxyl protected dimer 'Boc-VA-OMe' was found at 1.37 ppm. The amine functional groups of the dimer moved up-field to the 5.0-5.3 ppm range from the 7.5-8.0 ppm range, after the coupling reaction due to the change in the neighborhood.

Infrared spectra of trimers have stronger amide peaks owing to the increased number of peptide bonds. The carbonyls, of the Boc 'C7[#]' protected amino acids have IR peaks in the wave-number regions of 1,395-1,365 cm⁻¹ and methyl groups in the 1,10-1,150 cm⁻¹ and 1,250-1,300 cm⁻¹ region.

Important points to note for the Boc VAV-OMe are the ester groups that are discernable in wave-number ranges of 1,700-1,750 cm⁻¹ and 1,150-1,110 cm⁻¹. The esters and amide bonds are masked by the large side groups as the peptide increases in size. This was thought to be the case because the bonds formed when the ester and amide are synthesized are shorter therefore an increase in length of the peptide, through coupling, masks them and makes them less visible in the IR spectrum.

¹H NMR peaks corresponding to the methyl ester CH₃-O, '4*' can be found between 3-4 ppm and NH₄⁺ 'between 7-12 ppm. The peaks which represent the amide bonds of peptides with methyl esters, '4*' are further downfield (7-11 ppm) in comparison to the amines which are found at (2-3 ppm),

owing to the protonation of the amine by hydrochloric acid. The tri-methyl groups of the 'boc' protecting groups are found in the range of 1.3-1.5 ppm and the ester group that protects the carboxylic acids in the range of 3.5-3.7 ppm. The methyl groups of Boc-VA-OMe are expressed at 3.7 ppm and for Boc-VAV-OMe at 3.8 ppm.

De-protecting BocVAV-OMe gave Boc-VAV-OH which had an elevated melting point and increased values for R_f in comparison to the unprotected trimer. The characterization of the de-protected Boc-VA-OH, using ESMS showed $311.15 (M)^+$, $431.17 (M + 5Na^+ + 6H^+)$, $631.28 (2M)^+$ sodium salts of Boc VA-OH. A successful de-protection was indicated by the absence of the O-CH₃ methyl 'C4*' protecting functional group in the IR-spectrum of Boc-GL-OH in the wave-number regions of 1445 and 1460 cm⁻¹.

There was little change in positions of the peaks, which represent the 'Boc' functional groups of the trimers, which are typically found in the ranges of 1.3-1.4 ppm. The characteristic peak for methyl-protecting group which is typically found in the range of 1445(s) and 1460(s) for Boc VAV-OMe, respectively, is absent after the de-esterification

Yields of the de-esterified dimers and trimers were greater than 60% with melting points that were generally higher than protected trimers. IR spectral data of de-protected trimer

'Boc VAV-OH,' shows were successful removal of the methyl-protecting group 'C4*' from the carboxylic acid, had occurred. The methyl-protecting group, which is typically reflected in the wave-number regions of 1310-1250 cm⁻¹, is absent.

¹HNMR experiments were run on the 400 and 600 MHz NMR instrument, using deuterated chloroform as solvent. The biggest difference between the spectral data of the protected trimers is the lack of the 'C4*/ 'CH₃ - O' methyl functional group, which protects the carboxylic acid.

Melting points of *N-tert*-butoxycarbonylanhydrides (NCAs') are generally lower than melting points of their precursors, possibly due to the packing of the rings. The ESMS spectra for the NCAs' were taken at a very low negative voltage to avoid splitting the rings. The spectrum comprises of various ions and salts of the structures and is shown in Figure 3. 'M' denotes the NCA. Chloride and sodium ions form salts with the NCA and their charges are indicated by their chemical symbols, below the spectrum in the subject line.

The IR spectrum of the NCA shows the fingerprints of the molecular structure and functional groups with slight shifts up-field, for the carbonyl and carbamate of wave-number ranges of 1,710-1,690 cm⁻¹, with strong CO-NH signals in 1220-1020 cm⁻¹ range. There was an absence or reduced signals for the amino acid in the wave-number regions of 3500 cm⁻¹, an absence of the 'Boc' protecting group, in the wave-number region of 1395-1385 cm⁻¹ and the presence of the anhydrides in the wave-number region of 1840-1740 cm⁻¹, in figure 1.

FTIR spectra of NCA VAV show the compound's functional groups where the carbonyls and carbamates shift down field to Wave number regions of 1,800-1,760 cm⁻¹ and 1,870-1,830 cm⁻¹, respectively.

NCA-VAV

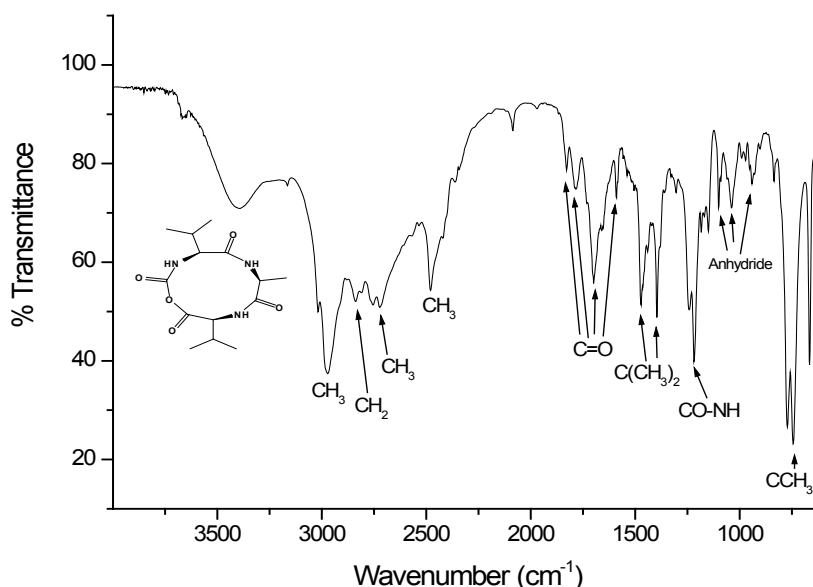


Figure 1. FT-IR NCA-VAV/ 6, 10-diisopropyl-3-methyl-1,4,7-triazecane-2,5,8,9 tetraone

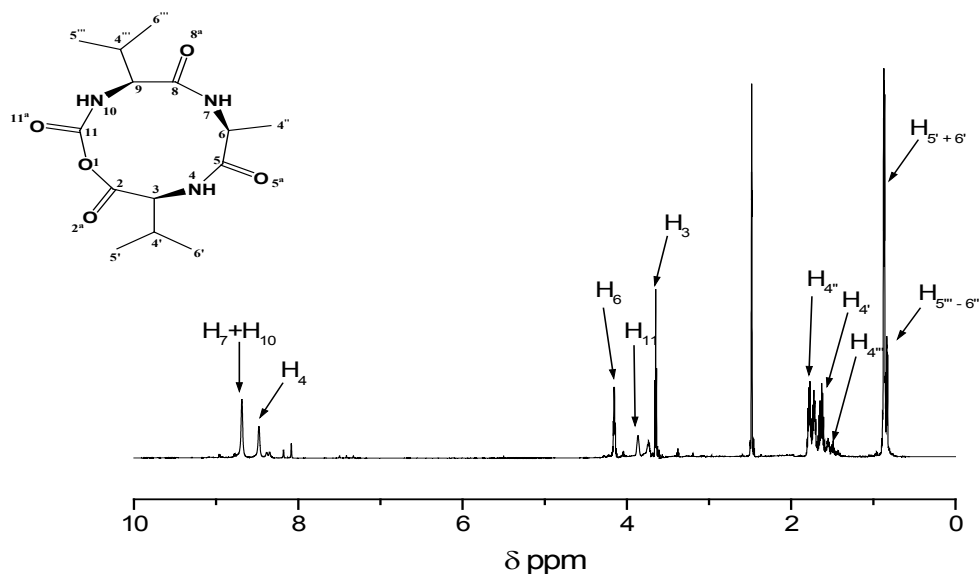
NCA-VAV

Figure 2. ^{13}C of NCA VAV/ 6, 10-diisopropyl-3-methyl-1,4,7-triazecane-2, 5, 8, 9-tetraone

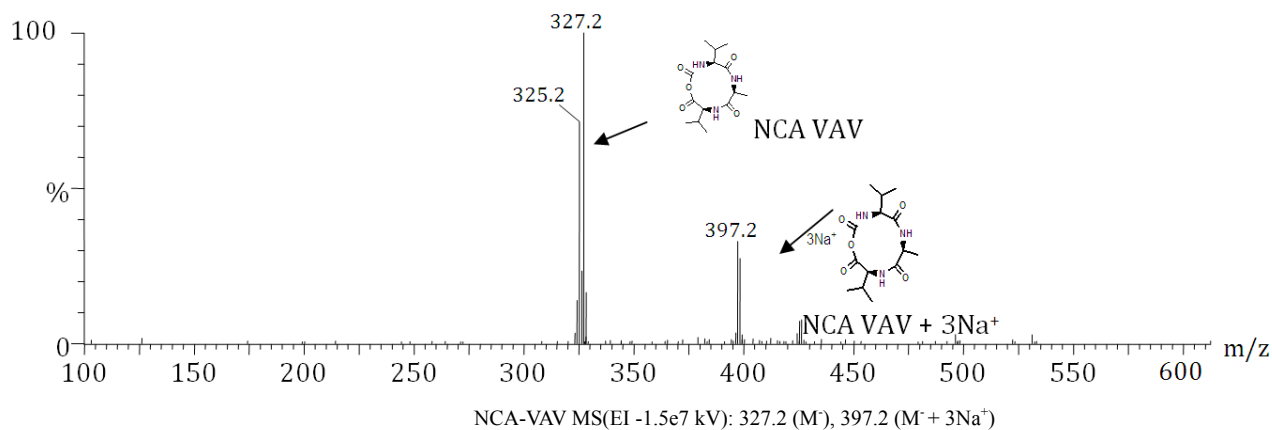


Figure 3. ESMS of NCA-VAV and its salts

Table 1. Reaction conditions of dimers to NCA dimers

NCA	Reaction conditions: solvent, reaction temp, time	Solvent front vs. solvent system values (R_f)	Yield (wt %)	Mp ($^{\circ}\text{C}$)
NCA-VAV	THF, 55 $^{\circ}\text{C}$, 4h 0 $^{\circ}\text{C}$ \rightarrow 30 $^{\circ}\text{C}$, 4h	2:1(EtOAc/hex) 0.4	80	120.8
NCA-SIK [28]	THF, 55 $^{\circ}\text{C}$, 4h 0 $^{\circ}\text{C}$ \rightarrow 30 $^{\circ}\text{C}$	2:1(EtOAc/hex) 0.36	82	109.0
NCA-KLK [29]	DMF, 55 $^{\circ}\text{C}$, 4h 0 $^{\circ}\text{C}$ \rightarrow 25 $^{\circ}\text{C}$, 4h	3: 1 (ACN/hex) 0.29	93	71.9

The major differences are the down-field shift of most peaks owing to the change in environment. ESMS spectra of NCA-VAV is presented in Figure 3.

The reaction proceeds when the acid chloride attacks the acid group to form a halogen group, which in turn attacks the

carbonyl oxygen of the *tert*-butyl group. The rate-determining step is thought to be the nucleophilic attack of the halogen ion on the alkoxy group.

However, the use of acid halides such as phosphorus penta-chloride were found to cyclize free amino acids more

readily than the acid chlorides. [26] See Scheme 1.1. This hasn't been explored for dimers, trimers and small peptides. Table 1: shows the reaction conditions of NCA dimers

Factors such as the solvent / solvent mixture, type of proton grabber, as well as the Lewis acid used, were amended in attempts to obtain higher yields of NCA. Oxalyl chloride was considered to be the best Lewis acid for NCA s' VAV and SIK, and DIPEA the best proton grabber. [27] Oxalyl chloride is a liquid that yields gaseous by-products. Temperatures were kept as low as -20°C for the first hour of the reaction and gradually brought to room temperature thereafter, for both trimers NCAs. The NCA trimers needed some heat to push the reaction forward; however they gave higher yields with longer bench standing times.

As stated earlier, synthesis of stereo-regular polypeptides is advantageous because the nature of their polymers offers some control of physiological properties of specific bio-systems. This is a step closer to preparing synthetic proteins with specific repeat sequences. Proteins of known sequence such as SIKVAV, an active laminin of most extra-cellular matrices is found in most tissues and encourages cell adhesion, migration and vascularization, in the body.

The biggest difference between the spectral data of the protected trimers precursors and NCAs' is the lack of the 'C4*/ 'CH₃ - O' methyl functional group, which protects the carboxylic acid. Melting points of N-*tert*-butoxycarbonylanhydrides (NCAs) are generally lower than melting points of their precursors, possibly due to the closer packing of the rings (sp³ hybrid orbitals) and hence an augmented delocalization, which makes the NCAs compounds more labile.

4. Experimental

4.1. Equipment

Perkin Elmer melting point apparatus (DMA8000), nexus FTIR, Oxford NMR model (Agilent), CapLC coupled to Q-TOF Ultima ESMS.

All L-amino acids and peptides were characterized by their melting points and R_f values (product front vs. solvent front), and by nexus FTIR, Oxford-NMR (300-600 MHz) and Proteomics using a CapLC coupled with a Q-TOF Ultima ESMS. The melting points were observed with Perkin Elmer, melting point instrument (DMA 8000). Product front vs. solvent front was obtained from thin layer chromatography (TLC) using appropriate solvent systems. Nicolet Nexus Fourier-Transform infrared (FTIR) was used

to characterize 'fingerprint' spectra of organic molecules. A nexus FTIR instrument was used under inert conditions to improve the signal: noise ratios, and obtain better spectra. All the above instruments are at The University of Stellenbosch, South Africa. Molecular masses were obtained using time-of-flight electro-spray mass spectrometry (TOF-ESMS). [30, 31] Ionization by spray techniques are based on desorption of the ions of a compound from a chromatographically separated liquid solution, to a mobile phase that contains an electrolyte.

4.2. Materials

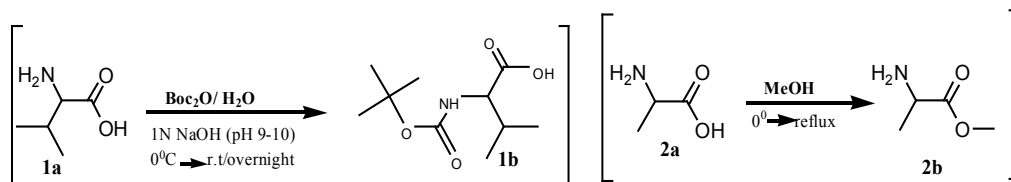
Alanine, valine, thionyl chloride, oxalyl chloride, methanol, 4-*tert*-butoxycarbonyl, lithium hydroxide, potassium sulphate, magnesium sulphate, sodium bicarbonate and sodium hydroxide were obtained from Sigma Aldrich; hydroxybenzotriazole (HbT), silica gel, O-(Benzotriazole-1-yl)-N'-N'-N'-tetramethyluronium tetrafluoroborate (TBTU) were obtained from Advanced ChemTech, South Africa and were used without further purification. THF, DMF, acetonitrile and ethanoic acid, diisopropyl ethylamine were purified by re-distillation before use. Water was obtained from an on site distilling and de-ionizing system.

4.3. Procedure

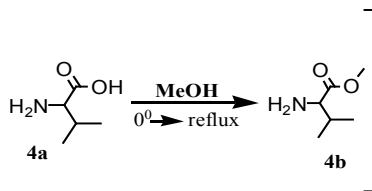
The amine functional group of the first L-Valine (**1A**) was protected with 4-*tert*-butoxycarbonyl (boc) [32] [33] to form a Boc-Val (**1B**). [34] The crude product was purified by chromatography over silica gel (0.06nm, DCM: MeOH), R_f = 0.28, and gave white crystals of m.p. 97°C. [35] (Yield of 62%, see Figure 2).

The other amines (**2A** and **4A**) L-Valine and L-Alanine were protected with methoxy groups in a solution of thionyl chloride and methanol, at 0°C. [36] The reaction afforded clear white solid crystals of methyl 2-aminopropanoate/ A-OMe, (m.p of 108°C, yields of 66%). HPLC showed purity of 99%; TLC Chromatogram-DCM: MeOH, 20:1, R_f = 0.2. Methyl 2-amino-3-methylbutanoate/ H-V-OMe gave yields of: 94%, mp = 98.8°C, TLC Chromatogram-DCM: MeOH (20:1) R_f = 0.65.

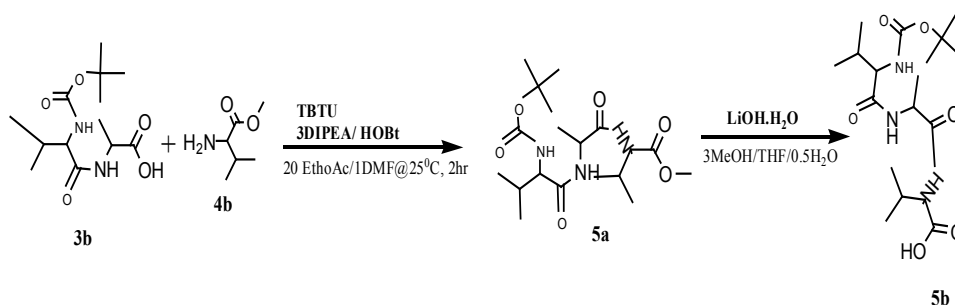
The, **2B** gave yields = 81.1%, M.P. = 108°C, Chromatogram: 20DCM: 1MeOH, R_f=0.2 [37], **4B** [38], which gave a yield = 94%, M.P = 98.8°C, Chromatogram: 20DCM: 1MeOH, R_f = 0.65. The protected amines were then coupled with TBTU [39] in an alkaline solution, to form protected dimer (**3A**) and gave yield = 94%, M.P. = 98.8°C, Chromatogram: 20DCM:1MeOH, R_f = 0.75. [40] and trimers (**5A**), as described in scheme 1.5. [42]



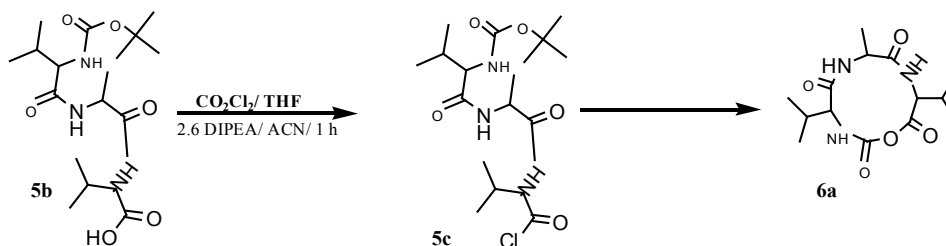
Scheme 1.3. Protecting amine groups on amino acids using a *tert*-butoxycarbonyl for the amine and methoxy for the carbonyl



Scheme 1.4. Protecting carboxylic functional group with methoxy group



Scheme 1.5. Coupling of partially protected dimer Boc Val-Ala-OH and Val-OMe then de-protected to give Boc Val-Ala-Val-OH. [42]



Scheme 1.6. Cyclisation of trimer to NCA VAV

This protected compound, '5A,' was de-protected with hydrated lithium hydroxide to free it for coupling (5B [43]) and gave [1] Yield = 94%, M. P. = 123.5°C, Chromatogram: 20DCM: 1MeOH, R_f = 0.39. Followed by the cyclisation (5B). [44] '3A' which was then dried over anhydrous magnesium sulphate, filtered, further purified by column chromatography and vacuum dried to give a pure white solid. HPLC gave a purity of 95%, a yield of 85%, mp of 98.5°C and TLC chromatography DCM: MeOH, 20:1, R_f = 0.75. The synthesis of Boc-Val-Ala-OH/ 2-(2-(tert-butoxycarbonyl)-3-methylbutanamido) propanoic acid (5B) gave: a Yield of 94%, mp = 123.5°C, TLC Chromatogram in DCM:MeOH ratio (20:1) and R_f = 0.39.

A solution of Boc-Val-Ala-OH (1.45 g, 4.301 mmol) '3B' and a coupling agent O-(Benzotriazole-1-yl)-N,N',N'-tetramethyluronium tetrafluoroborate (TBTU) (1.38 g, 4.301 mmol) were stirred in a solution of (DMF/EtOAc) of ratio(20:1) for 5-10 min. A separate solution of hydroxybenzotriazole (HoBt) (0.582 g, 4.301 mmols) in 5 ml of a 20:1 solution of (DMF/ EtOAc) was added to the first mixture and stirred continuously for a further 10 -15 minutes. After complete dissolution, diisopropyl ethylamine (DIPEA) (0.54 ml, 4.301 mmol) and H-Val-OMe (0.54 g, 4.301 mmol) were added to the reaction flask and the stirring was continued until complete dissolution. After three hours, ethyl acetate (10 ml) was added into the reaction flask and the mixture was cooled to 0°C, washed with 5% KHSO_4 then 5% NaHCO_3 , and then washed with a saturated solution of

sodium chloride. The product 'Boc VAV-OMe' (5A) was then dried over anhydrous magnesium sulphate, filtered and further purified by column chromatography then vacuum dried to give a pure white solid with HPLC purity of 95%, a yield of 85%, m.p. of 73.4°C and, an R_f = 0.65 in DCM: MeOH, 20:1.

The protected trimer was then freed with hydrated lithium hydroxide, as described earlier, to afford Boc Val-Ala-Val-OH, '5B'.

Cyclisation of 6, 10-diisopropyl-3-methyl-1,4,7-triazecan e-2,5,8,9-tetraone/ NCA-VAV

A solution of freshly distilled THF (10 ml, 7.59 mmol) and Boc-VAV-OH (1g. 2.58 mmol) were cooled to (-20 °C). (0.48 ml, 7.59 mmol) of oxalyl chloride was added whilst stirring the mixture for 30mins. Then a solution of (1.32 ml, 2.58 mmol) DIPEA in acetonitrile (2ml) was added to the flask and stirring continued. The content of the reaction flask was stirred for 2h at -20°C and was then allowed to warm up to room temperature (over a period of 1h) and stirred for a further 4h. The reaction was quenched, by pouring the mixture, onto ice. The product was extracted into ethyl acetate (3X10 ml) and dried over MgSO_4 . The filtrate was dried under vacuum to afford the desired NCA-VAV crystals. Re-crystallization from ethyl acetate gave a yield of 80% and a melting point of 120.8°C. [45] Cyclisation of NCA KLK was carried out in the same way and full characterization are listed in the results section.

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Abbreviations

2-(2-(2-(tert-butoxycarbonyl)methylbutanamido)propanamido)-3-methylbutanoate = Boc-VAV-OMe,
2-(2-(2-(tert-butoxycarbonyl)methylbutanamido)propanamido)-3-methylbutanoic acid = Boc-VAV-OH; 6, 10-diisopropyl-3-methyl-1,4,7-triazecane-2,5,8,9-tetraone = NCA VAV

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