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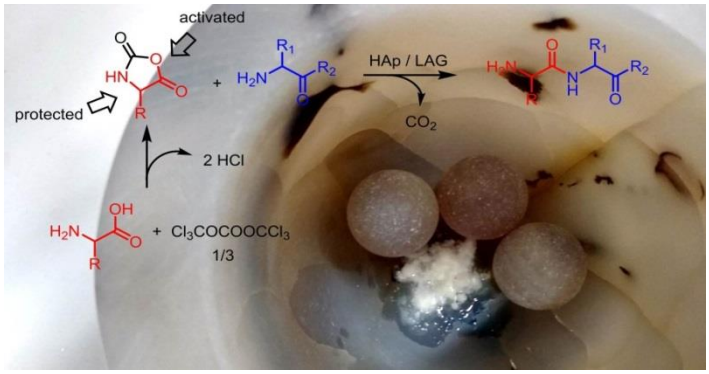
Abstract

Peptide bond forming reactions are fundamental to the synthesis of peptide s and peptidomimetics. Unfortunately, the chemistry of the peptide bond rouses much concern in terms of economic cost, environmental impact, and production of wastes, especially toxic organic solvents. In a Green Chemistry perspective, we reconsidered the use of unprotected N-carboxy anhydrides (NCAs) of standard α-amino acids under minimal liquid-assisted grinding (LAG) conditions. NCAs are very attractive in peptide synthesis for their simplicity and atom economy. Indeed, the molecular bulk of these N-protected/C-activated bidentate reactants remains with the newly formed peptide. However, the NCAs are prone to polymerization, a formidable obstacle to the development of mechanochemical conditions. Nevertheless, polymerization can be prevented by operating under controlled basic conditions. In this work, we describe peptide bond forming reactions from NCAs and amino partners by LAG in amalgam with nanocrystalline hydroxyapatite (HAp) powder as a bio-compatible, reusable inorganic base. The experiments supported the combination of HAp and the green solvent γ-valerolactone, and highlighted the importance of NCAs' purity and crystallinity, as studied by NMR, SEM, and XRD. The efficacy of the procedure was assessed by the synthesis of the 13-mer cytotoxic peptide PTP7.



Keywords: liquid-assisted grinding; recyclable base; inorganic biocompatible base; green solvents; peptide bond; peptides; amide bond; γ-valerolactone; nanocrystals; PTP7.

1. Introduction[1]

In the last decade, the peptide therapeutics market experienced a significant growth, and is expected to continue its momentum and expand over the coming years, with a predicted Compound Annual Growth Rate of 9.4%, reaching the value of USD *48.04 billion* by 2025.[1] Compared with small organic drugs, peptides generally offer greater efficacy and selectivity, and reduced systemic toxicity. Compared with biologics, i.e. proteins and monoclonal antibodies (Abs), peptides have greater stability and bioavailability, and reduced immunogenicity. Besides, peptides have way lower manufacturing costs, and lower royalty stack because of a simpler intellectual property.[2]

From a structural point of view, the oligopeptides approved by FDA in the last couple of years cover the whole spectrum of complexity.[3,4] Angiotensin II is an endogenous linear octapeptide for the control of blood pressure. Etelcalcetide, for the treatment of secondary hyperparathyroidism, includes a chain of seven D-amino acids. Plecanatide, a cyclopeptide with two disulfide bridges, is indicated for the treatment of chronic idiopathic constipation. Abaloparatide, for the treatment of osteoporosis, contains an aminoisobutyric residue. Semaglutide is a PEG-modified human glucagon-like peptide-1 (GLP-1) analog for the treatment of type 2 diabetes mellitus. The pseudo-tripeptide macimorelin is used for the treatment of adult growth hormone deficiency. Lu 177 DOTA-TATE contains the cyclic octapeptide Tyr^3-octeotride, and is

[1] **Abbreviations**: NCA, N-carboxy anhydride; HAp, hydroxyapatite; Abs, antibodies; FDA, Food and Drug Administration; Cit, citrulline; UNCA, urea-*N*-carboxyanhydride; LAG, liquid-assisted grinding; Boc, tert-butyloxycarbonyl, Fmoc, fluorenylmethoxycarbonyl; EDC, *N*-(3 dimethylaminopropyl)-*N*'-ethylcarbodiimide; HOBt, hydroxybenzotriazole; MW, microwave; RP HPLC, reverse phase high-performance liquid chromatography; DAD, diode array detector; MSD, mass spectroscopy detector; NMR, nuclear magnetic resonance; DMSO, dimethylsulfoxyde; RT, room temperature; FTIR, Fourier transform infra-red spectroscopy; PXR, powder X-ray diffraction; SEM, scanning electron microscopy; ESI MS electrospray ionization mass spectroscopy; GVL, γ-valerolactone; DMSO, dimethylsulfoxyde; mw, molecular weight; UPLC, ultra-high performance liquid chromatography; HRMS, high resolution mass spectrometry.

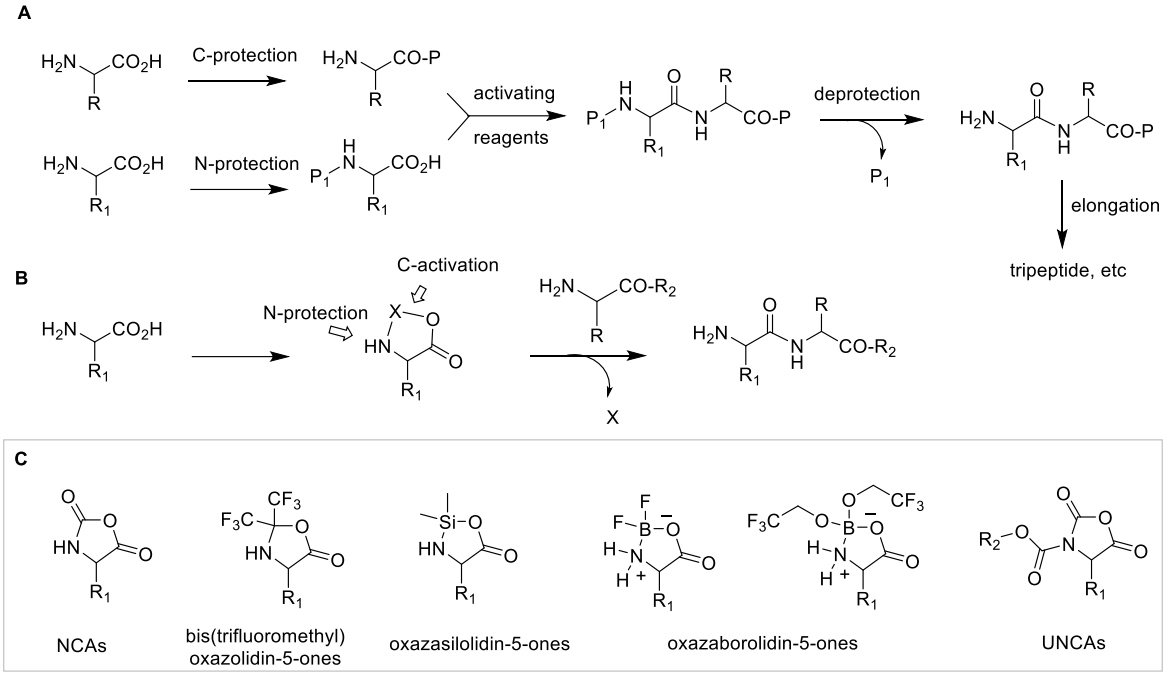
used for PET imaging and radionuclide therapy. Enfortumab vedotin and polatuzumab vedotin are monoclonal Abs conjugated to monomethyl auristatin E via a Val-Cit cleavable linker, indicated for urothelial cancers. The 13-mer lineal peptide afamelanotide is a potent α-melanocyte-stimulating hormone (αMSH) analogue. Bremelanotide is a hepta side-chain to side-chain cyclic peptide utilized for the treatment of hypoactive premenopausal women. Finally, several hybrid peptidomimetics have been also approved, e.g. velpatasvir, voxilaprevir, glecaprevir, pibrentasvir, which are composed of di- or tripeptide sequences attached to non-peptidic scaffolds.[3,4,5]

Despite their tremendous therapeutic potential,[6] the utilization of peptidic drugs is still limited by their expensive production and challenging purification.[7,8,9] Short and medium size peptides are usually prepared by chemical synthesis, in solution or in the solid phase (Scheme 1).[10,11] Both methods require an excess of amino acids and of activating agents,[12] and extensive protection/deprotection strategies.[13] In particular, each coupling/deprotection cycle generally needs large volumes of hazardous organic solvents, producing a huge amount of wastes.[14] Dichloromethane (DCM), dimethylformamide (DMF), or *N*-methylpyrrolidone (NMP), are toxic organic solvents commonly utilized in peptide synthesis. In particular, DMF has been included by the European Chemicals Agency in the candidate list for authorization as Substance of very high concern (SVHC), for which a restricted use in the near future is expected (inclusion in Annex XIV of REACH, "Authorization List").

According to the principles of Green Chemistry,[15] the activating agents and the protecting groups to be utilized can be reconsidered in terms of atom economy. Of particular interest are the bidentate derivatives of amino acids that simultaneously protect the α-amino group and activate the carboxy group toward nucleophiles (Scheme 1). A century ago, Leuchs employed phosgene or derivatives thereof to generate reactive *N*-carboxyanhydrides (NCAs) for subsequent reaction with amines.[16] In the period 1950-60 Bartlett and Hirschmann explored the coupling of NCAs to amino acids.[17] Additional *N*-protection of NCAs yielded the urea-*N*-carboxyanhydrides (UNCAs).[18] More details on the chemistry of NCAs and UNCAs are given in the Results and Discussion. Spengler, Burger *et al.* reported the use of gaseous hexafluoroacetone (HFA) for the formation of oxazolidinones activated towards amines.[19] Liskamp *and co*. used dichloroalkyl silanes[20] or boron trifluoride[21] to produce activated Lewis acid conjugates in which the amino acid was proposed to coordinate in a bidentate fashion. Sheppard et al. described $B(OCH_2CF_3)_3$ and other Lewis acids, e.g. $Ti(OiPr)_4$, as effective reagents for the direct synthesis of peptides from unprotected amino acids and amines.[22]

Another opportunity for making peptide chemistry more sustainable is the drastic reduction of the involved volumes of solvents used.[23,24] in this respect, coupling reactions can be performed through flow chemistry, using mini or microreactors requiring minimal volumes of solvents.[25,26] Alternatively, mechano or mechanoenzymatic chemistry allows operating under (virtually) solvent-free conditions.[27]

Mechanochemical activation consents to obtain with complete preservation of stereochemistry also peptide fragments containing highly epimerization-prone and/or highly hindered amino acids at C-term.[28] To improve the homogeneity of the solid-state reactions, small amounts of solvents can be added (liquid-assisted grinding, LAG), resulting in increased yields and shorter reaction times.[29,30,31] Further, harmful standard organic solvents can be replaced by green alternatives, i.e. non-toxic, recyclable solvents obtainable from renewable feedstocks.[32,33,34] On the other hand, some protocols have been developed to perform peptide coupling between minimally protected amino acid in an aqueous[35] or biphasic[36] environment.



Scheme 1. (A) Classic peptide synthesis by protection/coupling/deprotection procedures, in solution; P, P_1, protecting groups. (B) Expedient synthesis using dually N-protected/C-activated bidentate derivatives; X, see examples in box C. (C) Examples of 5-membered bidentate heterocycles: NCA,[16,17] bis(trifluoromethyl)oxazolidin-5-ones,[19] oxazasilolidin-5-one,[20] oxazaborolidin-5-ones,[21] UNCAs.[18]

Finally, organic bases such as trimethylamine, *i*Pr_2NEt or lutidine, typically utilized in peptide synthesis, can be replaced by inorganic salts, for example, $NaHCO_3$, Cs_2CO_3, or hydrotalcite.[29,30] Very recently, we developed a mild mechanochemical solvent-free peptide bond-forming protocol using Boc- or Fmoc-protected amino acids and amino esters or amides· HCl salts, in an amalgam with low-melting[37] *N*-(3 dimethylaminopropyl)-*N'*-ethylcarbodiimide· HCl (EDC· HCl) and hydroxybenzotriazole (HOBt) as the coupling agents, and nanocrystalline hydroxyapatite (HAp) as a fully bio-compatible, reusable inorganic base.[38]

In this scenario, we decided to reconsider the potential use of unprotected NCAs of standard α-amino acids (Scheme 1) in the perspective of forming peptide bond under Green Chemistry conditions. The attractiveness of peptide synthesis by NCAs lies in its amazing atom economy, since the molecular bulk of these N-protected/C-activated bidentate reactants remains in the product. Unfortunately, the NCAs are prone to polymerization; nevertheless, polymerization can be prevented by operating under strictly controlled basic conditions (see in the Results and Discussion).[39,40,41,42] Hence, we tested peptide bond formation by milling an amalgam composed of NCAs and amino esters or amides and an excess of nanocrystalline HAp.[38]

2. Materials and methods

General methods. All commercially available reagents purchased from Merck (Darmstadt, Germany) were used without further purification. The synthetic procedures under MW irradiation were performed with a Microwave Labstation for Synthesis (Micro-SYNTH, Bergamo, BG, IT) equipped with a built-in ATC-FO advanced fiber-optic automatic temperature control. Ball milling was carried out in a low-frequency PlanetaryMill Pulverisette (Fritsch GmbH, Idar-Oberstein, Germany), mounting an agate jar (ø = 7.5 cm) equipped with three balls of the same material (ø = 2.0 cm). Purities were assessed by analytical reverse phase high-performance liquid chromatography (RP HPLC) on a 1100 series apparatus (Agilent, CA, USA), using a XSelect Peptide CSH C18 column (Waters, Milford, MA, USA), 4.6 mm × 100 mm, 130 Å, 3.5 μm; DAD 210 nm; mobile phase: from 8:2 water/CH_3CN to 2:8 water/CH_3CN, in 8 min, at a flow rate of 0.5 ml min^{-1}, followed by 10 min at the same composition. ESI-MS was done on a MS single quadrupole HP 1100 MSD detector (Agilent). UPLC analysis was performed on an Acquity UPLC H-Class Plus apparatus, using a Acquity C18 BEH column, 1.7 μm and 2.1 x 100 mm; mobile phase: from 3:1 H_2O/0.1% HCO_2H//CH_3CN/0.1% HCO_2H to 1:1 H_2O/0.1% HCO_2H //CH_3CN/0.1% HCO_2H, in 1.5 min at a flow rate of 0.5 mL/min, followed by 1.0 min at the same composition, then to 1:4 H_2O/0.1% HCO_2H//CH_3CN/0.1% HCO_2H in 0.5 min, followed by 1.0 min at the same composition. High resolution mass spectrometry (HRMS) was performed with a Xevo G2XS QTof apparatus. ^1H NMR was performed at 400 MHz on a Varian Gemini 400 (Agilent) in 5 mm tubes in DMSO-d_6 at rt; chemical shifts are reported as δ values relative to residual H peak (δH = 2.50 ppm). NCAs morphology was observed using a Zeiss Leo1530 Gemini field-emission scanning electron microscope (SEM) equipped with InLens detector and operating at 5kV. Samples were sputter coated with gold before observation. Powder X-ray diffraction patterns were recorded using a PANalytical-X'Pert PRO powder diffractometer (Malvern Panalytical-Spectris, Egham, UK) equipped with a fast X'Celerator (Malvern Panalytical-Spectris) detector (λ = 0.154 nm, 40 mA, 40 kV). For phase identification, the 2θ range was investigated from 10 to 60 2θ° with a step size of 0.1° and time/step of 100 s.

General protocol for peptide bond formation. The NCA (0.2 mmol) was mixed with the amino partner·HCl (0.2 mmol), HAp powder (100 mg) and γ-valerolactone (GVL) (0.2 mL). The mixture was milled

at 10 Hz frequency into agate jar equipped with three balls. The milling system (jar and balls) was precooled at +3°C into a regular fridge. After 30 min, milling was stopped, the mixture was diluted with ethanol (5 mL), and the suspension was separated by centrifugation. The HAp crystals were washed twice with ethanol (5 mL) and collected by centrifuge, and dried at 50°C for 6h. The collected organic layers were treated with 1M HCl in ethanol (50 μL), and stirred for 30 min at RT, the solvent was removed at reduced pressure, and the resulting dipeptide·HCl salt was triturated with EtOAc.

3. Results and Discussion

In terms of atom economy, unprotected NCAs represent the most convenient amino acid derivative to perform peptide synthesis, provided that expedient reaction conditions could be designed. Indeed, NCAs are prone to polymerization in a neutral or acidic environment, due to the decarboxylation of the intermediate carbamic acid (Scheme 2). Hence, the reaction conditions to the synthesis of a dipeptide must be carefully controlled. The original Hirschmann's NCA process involved the batch reaction of α-amino carboxylates with solid NCAs in 1M sodium borate buffer. The pH was maintained at 10.2 by addition of 6M KOH. Hirschmann recognized the need for high shear mixing to rapidly disperse the highly reactive solid NCA. To solve this practical aspect, solid NCA was added portionwise to the reaction into a kitchen blender, and temperature was kept at 0°C by adding ice into the shake.[17]

Several modifications of this original procedure have been proposed, including the use of organic acids and bases.[43] NCAs were also utilized in solid phase peptide synthesis on PEG-based ChemMatrix® resins under controlled aqueous conditions, i.e. in borate buffer pH 10.2 at 5 °C. This procedure afforded with sufficient yield and purity a short peptide amide, without the need of coupling reagents nor protecting groups.[35]

A more recent study analyzed in details the mechanisms, the kinetics, and the many parameters, e.g. stirring and fluid velocity, particle shape and size, temperature, pH, of the coupling reactions in the liquid boundary layer and bulk solution during dissolution of the solid NCA, responsible for dimerization or polymerization, or by-product formation.[44] Control of the conditions appeared to be important to prevent side reactions (e.g. NCA hydrolysis or decarboxylation). This comprehensive study led to the construction of a continuous flow stirred tank reactor designed to control, within a narrow range, operating conditions that favor the reaction of NCA with an amino acid and minimize side reactions.[44]

In summary, the complex control of the reaction parameters necessary to avoid polymerization strongly diminishes the practical utility of NCAs in peptide synthesis. As a matter of fact, NCAs are more frequently utilized for the fabrication of high molecular weight polypeptides via ring-opening polymerization.[42,56,58] In order to definitively prevent the risk of polymerization, but at the disadvantage of atom economy, Fuller et al. proposed *N*-protection of NCAs with Boc or Fmoc to give UNCAs (Scheme 1).[18] Recently, Lamaty and co.

utilized UNCAs of α-amino acids and α- or β-amino ester hydrochloride counterparts in the presence of $NaHCO_3$, for the solvent-free preparation of dipeptides, including aspartame, into a steel high-frequency ball-mill.[45] The same authors improved the procedure by adding minimal amounts of environmentally benign solvents (EtOAc or tBuOAc).[29] Subsequently, Juaristi et al. successfully utilized N-Boc-UNCAs derived from β-amino acids to perform the solvent-free coupling with different α- and β-amino ester hydrochlorides under ball-milling activation.[46]

In this context, we explored the reactions of unprotected NCAs with amino counterparts in LAG conditions, in amalgam with nanocrystalline hydroxyapatite (HAp). HAp, $Ca_{10}(PO_4)_6(OH)_2$, is a common form of calcium phosphate structurally similar to the mineral phase of bone tissues. We choose HAp for its great bio-compatibility, high affinity for amino acids, low solubility, high stability, and for its relatively weak basic character that may prevent unwanted reactions.[38,47] The properties of HAp vary depending on the method of preparation, thus enabling their use as nanomaterials, for diverse applications in the biomedical field.[48,49] In contrast to hydrotalcite,[29,30] the powder X-ray diffraction (PXRD) of HAp shows a set of intense and separate peaks (Figure 43), suggestive of higher crystallinity.[50] In this work, the HAp nanocrystals were synthesized by a co-precipitation method, and characterized by PXRD, Fourier transform IR (FT IR) (Supporting Information), Transmission electron microscopy (TEM). In particular, the TEM micrograph confirmed the presence of plate-shaped HAp crystals with mean dimensions of about 100 nm × 30 nm (Figure 43).[38]

Initially, the reaction conditions were explored for optimization using as the model partners D-Val-OMe·HCl and the NCA of Trp (TrpNCA). The latter was prepared by means of a modified version of the Fuchs-Farthing method.[51] A limp mixture of the amino acid and one third of $OC(OCCl_3)_2$ (triphosgene) was mixed under MW irradiation while gently blowing with nitrogen (Scheme 2).[35] Though included in the hazardous compounds (GHS05, GHS06), triphosgene is regarded as a convenient alternative to phosgene, provided that it was properly handled.[52]

Several authors pointed at the purity and the crystallinity of the NCAs as fundamental issues for increasing the quality of their reactions.[40,53,54,55,56] Typically, NCAs contain traces of water, acid, acid chlorides, or isocyanates. The presence of minute impurities can cause problems by promoting side reactions. Hence, we utilized diverse batches of TrpNCA isolated according to alternative protocols, i.e. (A) by precipitation from THF/hexane (TrpNCA-A),[35] or (B) by the procedure recommended by Semple et al. (TrpNCA-B, Experimental Section);[53] finally, (C) after Semple's procedure, the NCA was subjected to repeated crystallization from THF/hexane upon slow decrease in solubility at low temperature (TrpNCA-C).[57] The resulting samples were analyzed by ^{1}H NMR spectroscopy, which confirmed the higher purity of TrpNCA-B and TrpNCA-C. The ^{1}H NMR spectra of TrpNCA-B and of the NCAs obtained from other amino acids, isolated according to method B, are shown in the Supporting Information.

Schäfer et al. proposed the recourse to PXRD for the analysis of NCAs.[40] Although this method does not provide structural information, sharp peaks characteristic for a crystalline phase are indicative of the crystalline state of the NCAs. Accordingly, we analyzed the NCA powders as obtained by the diverse protocols. The Powder X-ray diffraction patterns of the diverse samples of NCA, i.e. TrpNCA-A (Figure 1, red), TrpNCA-B (Figure 1, green), or TrpNCA-C (Figure 1, blue), showed slightly diverse diffraction patterns. Of note, the green line for TrpNCA-B is suggestive of a certain amount of amorphous material (Figure 1).

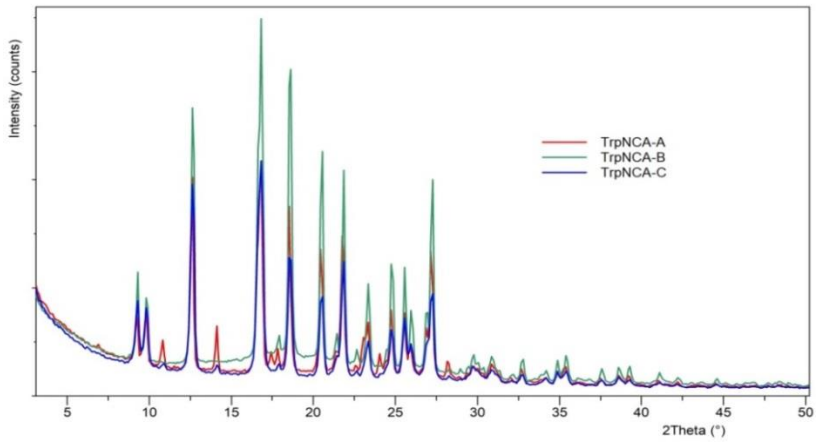


Figure 1. Powder X-ray diffraction patterns of TrpNCA isolated by precipitation (TrpNCA-A, red),[35] by the Semple's procedure (TrpNCA-B, green),[53] and by Semple's procedure/crystallization upon slow decrease of solubility (TrpNCA-C, blue).[57]

The crystal shapes of the NCA prepared by the three protocols were analyzed by SEM (Figure 2). The comparison of the images confirmed the diverse crystalline forms of the samples. TrpNCA-A, isolated by precipitation, showed disordered aggregates with no regular morphology and dimensions, which may be presumably due to the fast phase separation. In contrast, TrpNCA-B and TrpNCA-C presented well-defined separate crystals with different morphologies.

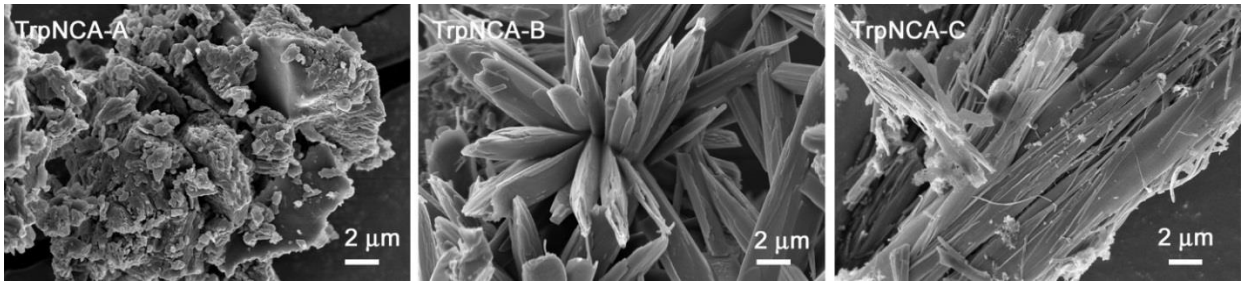
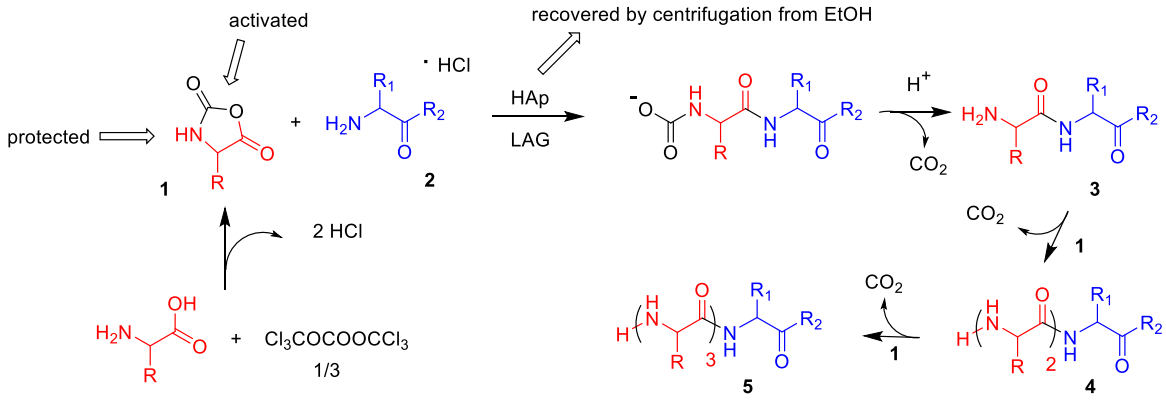


Figure 2. Scanning electron microscopy of TrpNCA-A, TrpNCA-B, TrpNCA-C showing alternative structures.

In particular, TrpNCA-B displayed rod-like particles composed by smaller aligned crystals elongated along a preferential direction; the rod-like particles were arranged from a common center to form

spherulites. TrpNCA-C sample obtained through crystallization by slow decrease of solubility was composed of long and flexible needle-like crystals with a very high length/width ratio.

In a set preliminary experiment (Scheme 2), a sample of TrpNCA (**1**) from each batch A-C (50 mg) was reacted with 1 equiv. of D-Val-OMe·HCl (**2**) in the presence of HAp (100 mg). The mixture was grinded into a low-frequency planetary ball mill, consisting of an agate jar equipped with three balls of the same material. The milling system (jar and balls) was precooled at +3°C into a regular fridge.[17] To increase the homogeneity of the mixture, the reaction was conducted in liquid-assisted grinding (LAG) conditions, i.e. in the presence of a minimal amount (0.1 mL) of the green solvent γ-valerolactone (GVL)[32-34] as a liquid additive (~~Figure 3~~Supporting Information).



Scheme 2. Synthesis of the NCAs (**1**) and peptide bond formation in the presence of HAp. The crude reaction mixtures containing the dipeptide **3** and possibly the unreacted **1** and the byproducts **4**, **5** were acetylated with Ac_2O prior to RP HPLC analysis (Supporting Information).

After 30 min, milling was stopped, the mixture was diluted with ethanol, and the suspension was separated by centrifugation. The HAp crystals were suspended in ethanol, collected by centrifuge, and dried in oven overnight. In the event that residual carbamate peptide intermediates were present, the collected organic layers were treated with 1M HCl in ethanol (50 μL),[17] and stirred for 30 min at RT (Scheme 2). The solvent was removed at reduced pressure, and the resulting dipeptide·HCl salt was triturated with EtOAc. Eventually, the residue was acetylated with an excess of Ac_2O in a mixture of dioxane and a saturated aqueous solution of $NaHCO_3$ (Supporting Information). We opted for this extra derivatization step for the convenience of the analysis of the mixtures (Table 1), which was thereafter performed by RP HPLC with DAD at 210 nM using a neutral mobile phase (water/acetonitrile). The RP HPLC analyses of the acetylated reaction mixtures confirmed that in all cases the conversion was almost quantitative (Supporting Information).

For the reaction of TrpNCA-A, electrospray ionization mass spectroscopy (ESI MS) confirmed the presence of the dipeptide Ac-Trp-D-Val-OMe (Ac-**3a**) as the major product (65%), but accompanied by a significant amount of the tripeptide Ac-$(Trp)_2$-D-Val-OMe (Ac-**4a**, 24%), and even of the tetrapeptide Ac-$(Trp)_3$-D-Val-OMe (Ac-**5a**). The acetylated reactant Ac-D-Val-OMe was also detected (Table 1, entry 1, and Supporting Information). Besides, the reaction gave rise to the formation of a number of minor by-products, possibly arising from decomposition and/or reactions of the NCA. Better results were observed in the reactions of TrpNCA-B or TrpNCA-C (Entry 2 and 3), under the same conditions described above, plausibly due to their higher purity as compared to TrpNCA-A. Interestingly, the best results were observed with TrpNCA-B. After acetylation, the analysis of the reaction revealed a higher yield of the dipeptide Ac-**3a** (79%), and reduced amounts of the acetylated reagent Ac-D-Val-OMe, of tripeptide Ac-**4a**, and other by-products, (Entry 2 and Supporting Information), while TrpNCA-C gave slightly inferior results (entry 3). The dependence of NCA reactivity in LAG conditions on a combination of purity and crystallinity was not completely unexpected. Schäfer et al. observed that highly crystalline NCA monomers gave higher mw chains by nucleophilic ring-opening polymerization.[40] Kanazawa et al. analyzed the crystal structure of amino acid NCAs by X-ray analysis to explain the reactivity in the solid state. Reproducibility could not be warranted in the polymerization of amino acid NCAs even when highly purified NCA crystals were used. The reactivity in the solid state was considered to depend also on crystal structure and density.[58]

Repeating the reaction with TrpNCA-B and a double amount of HAp (200 mg) and solvent (0.2 mL), allowed to further increase the yield of Ac-**3a** (93%), being the amounts of by-products almost negligible, plausibly due to an improved mixing of the reagents in the mill and a more homogeneous dough (Entry 4, Supporting Information). The replacement of GVL with other solvents gave diverse results. While EtOH gave dipeptide Ac-**3a** in modest yield and purity (Entry 5), DMSO gave a result comparable to GVL (entry 6). Even though not included among the prominent green solvents,[32-34] DMSO is regarded as an acceptable greener alternative for hazardous dipolar aprotics such as DMF.[32-34,59] However, due to the superior classification in the green solvent lists,[32-34] GVL~~LV~~ was designed as the solvent of choice.

Subsequently, using the TrpNCA-B/GVL combination under the best-performing conditions, HAp was replaced in the model reaction by other inorganic bases,[29,30] i.e. solid Cs_2CO_3, Al_2O_3, $Al(OH)_3$ in LAG conditions, as well by carbonate buffer pH 8; in all cases, the reaction gave comparatively inferior results (Entries 7-11).

Table 1. LAG of the NCAs and the amino ester/amide partners (HCl salts) in the presence of HAp and a green solvent. The crude reaction mixtures were acetylated prior to analysis.

entry	**1**	**2**·HCl	Base (x)[a]	Solvent (y)[b]		Ac-**3** (%)[c]	Ac-**4** (%)[c]	Ac-**5** (%)[c]	Ac-**1** (%)[c]

1	TrpNCA-A	D-Val-OMe	HAp (2)	GVL (2)	**a**	65	24	6	traces
2	TrpNCA-B	D-Val-OMe	HAp (2)	GVL (2)	**a**	79	12	5	Nd
3	TrpNCA-C	D-Val-OMe	HAp (2)	GVL (2)	**a**	76	15	6	Nd
4	TrpNCA-B	D-Val-OMe	HAp (4)	GVL (4)	**a**	93	traces	Nd	Nd
5	TrpNCA-B	D-Val-OMe	HAp (4)	EtOH (4)	**a**	80	8	traces	Nd
6	TrpNCA-B	D-Val-OMe	HAp (4)	DMSO (4)	**a**	92	traces	traces	Nd
7	TrpNCA-B	D-Val-OMe	Cs_2CO_3 (4)	GVL (4)	**a**	80	12	4	traces
8	TrpNCA-B	D-Val-OMe	Al_2O_3 (4)	GVL (4)	**a**	82	10	traces	traces
9	TrpNCA-B	D-Val-OMe	Cs_2CO_3 (4)	DMSO (4)	**a**	84	5	traces	Nd
10	TrpNCA-B	D-Val-OMe	$Al(OH)_3$ (4)	GVL (4)	**a**	78	10	traces	traces
11	TrpNCA-B	D-Val-OMe	carbonate buffer pH 8 (8)		**a**	83	9	traces	traces
12	TrpNCA-B	D-Val-OMe	HAp[d] (4)	GVL (4)	**a**	88[e]	traces	traces	Nd
13	TrpNCA-B	Phe-NH_2	HAp (4)	GVL (4)	**b**	90	5	Nd	Nd
14	AlaNCA	Phe-OEt	HAp (4)	GVL (4)	**c**	91	traces	Nd	traces
15	LeuNCA	Phe-OEt	HAp (4)	GVL (4)	**d**	90	6	traces	Nd
16	Lys(Cbz)NCA	Gly-OEt	HAp (4)	GVL (4)	**e**	86	traces	traces	traces
17	GluNCA	Phe-OEt	HAp (4)	GVL (4)	**f**	92	traces	Nd	Nd
18	TrpNCA	Pro-OBn	HAp (4)	GVL/(4)~~0.2~~	**g**	98	Nd	Nd	Nd
19	TyrNCA	Phe-OEt	HAp (4)	GVL (4)	**h**	90	7	Nd	Nd
20	PheNCA	Met-OMe	HAp (4)	GVL (4)	**i**	94	traces	Nd	Nd
21	PhgNCA	D-Val-OMe	HAp (4)	GVL (4)	**l**	93[f]	5[g]	traces	Nd

[a] x = mg HAp/mg NCA. [b] y = μL solvent/mg NCA. [c] Determined by RP HPLC. [d] Reutilized for up to 5 overall cycles. [e] Analysis of the 5th cycle. [f] 92:8 Ac-Phg-D-Val-OMe/Ac-D-Phg-D-Val-OMe. [g] Mixture of diasteroisomers. Traces, ≤ 4%. Nd, not detected.

After each reaction, the mineral HAp powder was recovered almost quantitatively by centrifugation, then it was cleaned up by washes with EtOH, and dryed for 6 h at 50◦C. The powder was reutilized for 4 further reactions cycles of the reaction between TrpNCA and D-Val-OMe, giving comparable results (Entry 12). The X-ray diffraction analysis (Figure ~~4A~~3A) of recovered HAp showed the same patterns as the pristine powder,[38] and TEM (Figure ~~4B~~3B) confirmed that the nanocrystals maintained the original shape and dimensions.[38]

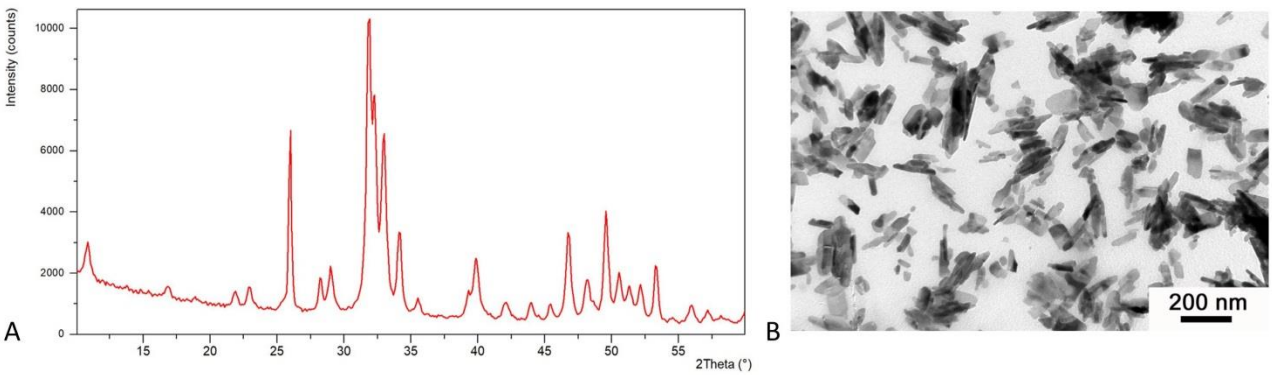


Figure 43. Characterization of HAp nanocrystals after five cycles of peptide bond formation. (A) Powder X-ray diffraction patterns. (B) TEM image.

Finally, the FT IR spectrum of the recycled material (Supporting Information) was the same of the pristine material without extra peaks, indicating the absence of inclusions of amino acids or other organic molecules.[38] In summary, the morphology and dimensions of the crystals after 5 overall cycles appeared to be preserved.

To confirm the amino acid scope of the procedure, the conditions reported in entry 4 were exploited for the preparation of dipeptides **3b-l** by reaction of amino amides or esters with diverse NCAs, prepared in turn by MW activation,[35] and subsequently isolated according to the Semple's protocol (B).[53] Also these NCAs were analyzed by ^{1}H NMR spectroscopy (Supporting Information). In all cases, after acetylation the coupling reactions gave results comparable to that of TrpNCA as reported in Entry 4 (Entries 13-21). Notably, the NCAs of Glu (entry 17) and Tyr (entry 19) have been utilized without protection at the side chain. The reaction between TrpNCA and Pro-OBn gave an outstanding 98% conversion to dipeptide (entry 1918). Possibly, the almost complete lack of by-products arising from NCA polymerization can be correlated to the higher nucleophilicity of the pyrrolidine ring of Pro-OBn respect to the primary amine of the dipeptide product H-Trp-Pro-OBn.[60]

In general, the peptide-forming reactions discussed above proceeded without appreciable epimerization, as determined on the basis of RP HPLC/ESI MS analyses (Supporting Information), which is in line with observations reported in the literature.[17,18,35,44] To assess the potential epimerisation of sensitive amino acids, the reaction was repeated on the racemisation vulnerable residue phenylglycine (Phg).[61] L-PhgNCA was prepared according to the literature (Supporting Information),[62] and utilized for reaction with D-Val-OMe under the usual conditions (entry 21). After acetylation, the analysis of the mixture revealed an amount of dipeptide Ac-**3l** in line with the other NCAs (93%), albeit in the form of a 92:8 mixture of the diasteroisomers Ac-Phg-D-Val-OMe / Ac-D-Phg-D-Val-OMe (Supporting Information), while the tripeptide Ac-**4l** was detected in traces as mixture of diastereoisomers Ac-(L/D-Phg)$_2$-D-Val-OMe.

Synthesis of the model cytotoxic 13-mer PTP7. In order to test the practical utility of the protocol described above, we prepared the 13-mer PTP7, a truncated derivative of the antimicrobial peptide gaegurin6 isolated from the frog *Rana rugosa*.[63] This peptide, of sequence H-Phe-Leu-Gly-Ala-Leu-Phe-Lys-Ala-Leu-Ser-Lys-Leu-Leu-OH, is of some interest for its significant toxicity against diverse cancer cell lines, i.e. human lung, prostate, breast and hepatocellular carcinomas. The peptide was prepared by reiteration of the usual protocol (Supporting Information). Ser was introduced as Ser(OtBu)NCA, and Lys as Lys(Boc)NCA. The intermediate peptide salts were crystallized from water/ethanol. The protecting groups at the side chains was removed with 0.5 M HCl in ethanol. The peptide PTP7 was obtained in reasonable

yield (55%); peptide purity was determined to be 76% by ultra-high performance liquid chromatography (UPLC), and its identity was confirmed by high resolution mass spectrometry (HRMS - ESI/QTOF) (Supporting Information). This result confirmed that the proposed protocol was almost as efficient as the conventional methods at room temperature on common polystyrene resins. Indeed, peptides between 10 and 20 residues long typically show UPLC purity in the 60-90% range, and 60-90% yield without purification.[64,65]

In conclusion, the protocol discussed herein represents a convenient method for the synthesis of oligopeptides, coherent to the principles of Green Chemistry. The use of NCAs allowed to avoid the recourse to protecting and activating agents. The reactions were conducted by grinding the reagents with the aid of a minimal amount of the green solvent GVL. The only solvents utilized in large amount were EtOH and EtOAc, ranked as "recommended" in the solvent selection guides.[32,59] On the other hand, an excess of HAp powder was utilized as base. However, this fully biocompatible base was easily recycled and reutilized, making the entire process convenient. In particular, HAp proved to be more efficient as compared to other inorganic bases in preventing NCA polymerization, generally regarded as the fundamental caveat to the use of NCAs for peptide synthesis. These results confirmed the efficacy of nanocrystalline HAp as an inorganic base in LAG conditions.[38] Interestingly, the analyses of diverse batches of NCA suggested that NCA purity and crystalline form significantly influenced the outcome of the reactions in terms of dipeptide versus polymer and by-products formation.

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Supplementary material. Experimental procedures: NCAs preparation and isolation; preparation of HAp nanocrystals; acetylation of crude reactions, and HPLC analysis. Figures S1-S11, ^{1}H NMR spectra of the NCAs isolated by method B. Figure S12, reaction mixture before and after grinding in an agate mill. Figures S13-S15, RP HPLC analyses of reactions between D-Val-OMe and TrpNCA. Figure S16-S24, RP HPLC analyses of reactions between amino partners and NCAs isolated according to protocol B. Figure S25, ultra HPLC analysis of PTP7 and high resolution mass spectrometry (HRMS-QTof). Figure S26, FIR adsorption spectra of recycled hydroxyapatite.

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