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An Optimized and Universal Protocol for the Synthesis of Morpholine-2,5-Diones from Natural Hydrophobic Amino Acids and Their Mixture

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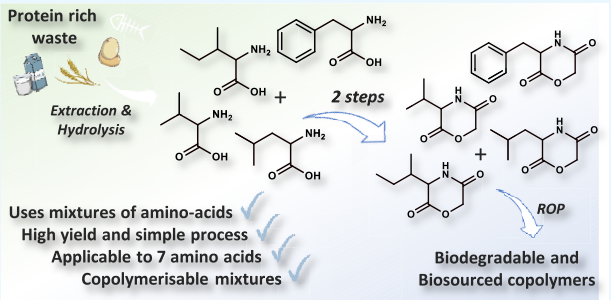
ABSTRACT: Morpholine-2,5-diones (MDs) are increasingly attractive compounds that can be produced using amino acid (AA) as a starting material. These compounds can undergo polymerization to produce biodegradable materials, namely, polydepsipeptides, that hold the potential to be used in medicinal applications. In this study, a simplified yet high-yield MD synthesis procedure was developed and applied to produce a range of MDs derived from hydrophobic AAs including Leu, Ile, Val, Phe, Asp(OBzl), Lys(Z), and Ser(tBu). Moreover, using a blend of hydrophobic amino acids (Leu, Ile, Val, and Phe), mixtures of MDs could be synthesized simultaneously. Finally, the polymerization of these MD mixtures was probed and proven successful. The concept investigated herein constitutes a novel path toward the valorization of protein-rich waste by producing renewable and biodegradable materials.



1. INTRODUCTION

Biomass is composed of different elements whose quantities vary depending on the raw material. Carbohydrates (cellulose, hemicellulose, starch, and lignin) are the most plentiful molecules followed by triglycerides (oils), proteins, and then terpenes.[1−4] Protein-rich wastes whose nutritional quality is insufficient or potentially harmful (slaughterhouse waste) contain interesting molecules (amino acids and oligopeptides) that chemists can separate and transform in biorefineries.[5] Indeed, amino acids (AAs) and oligopeptides can be transformed into numerous different compounds such as amines, nitriles, or dicarboxylic acids via biochemicals processes, some of which present real potential as building blocks for polymer synthesis.[5−7] For the valorization of such biosourced amino acids, it is necessary to extract the protein-rich materials, perform their hydrolysis, and then isolate and fraction the resulting individual AAs. Due to the large spectrum of physicochemical properties, fractioning AAs in aqueous solution is arguably the most difficult step in this process.[5−8] The separation of different AAs is laborious and is generally performed by taking advantage of the differences in their physicochemical properties such as hydrophilicity, charge, or pH-related solubility.[9,10] Therefore, it seems more interesting to obtain fractions of AAs with similar properties so that they can then be used in mixtures for potentially useful applications. Only mixtures of hydrophobic AAs are thus targeted in this study because hydrophobic AAs constitute a fraction that can be easily obtained after hydrolysis. Hence, a process wherein AAs can be upcycled while bypassing the AA fractioning phase would be an important step forward toward the use of biosourced materials. Among the polymers that can be produced using AAs, poly(esteramide)s (PEAs) are particularly attractive as they combine the great thermal and mechanical properties of polyamides with the biocompatibility and biodegradability of polyesters.[11−14] One category of such polyesteramides is polydepsipeptides (PDPs) that are essentially alternating motifs of α-amino acids and α-hydroxyacids. A number of studies have recently referred to the use of PDPs in the biomedical field, which is looking for innovative systems that can be applied for drug delivery, gene therapy, or tissue engineering. Thus, the combination of ester and amide linkages in the same polymer may open up prospects for the design of new materials with different properties (e.g., thermomechanical and degradability) used in the biomedical area.[11,12,14] PDPs can be produced via the ring-opening polymerization (ROP) of morpholine-2,5-diones (MDs), a cyclic monomer derived from AAs (Figure 1A).[15−20]

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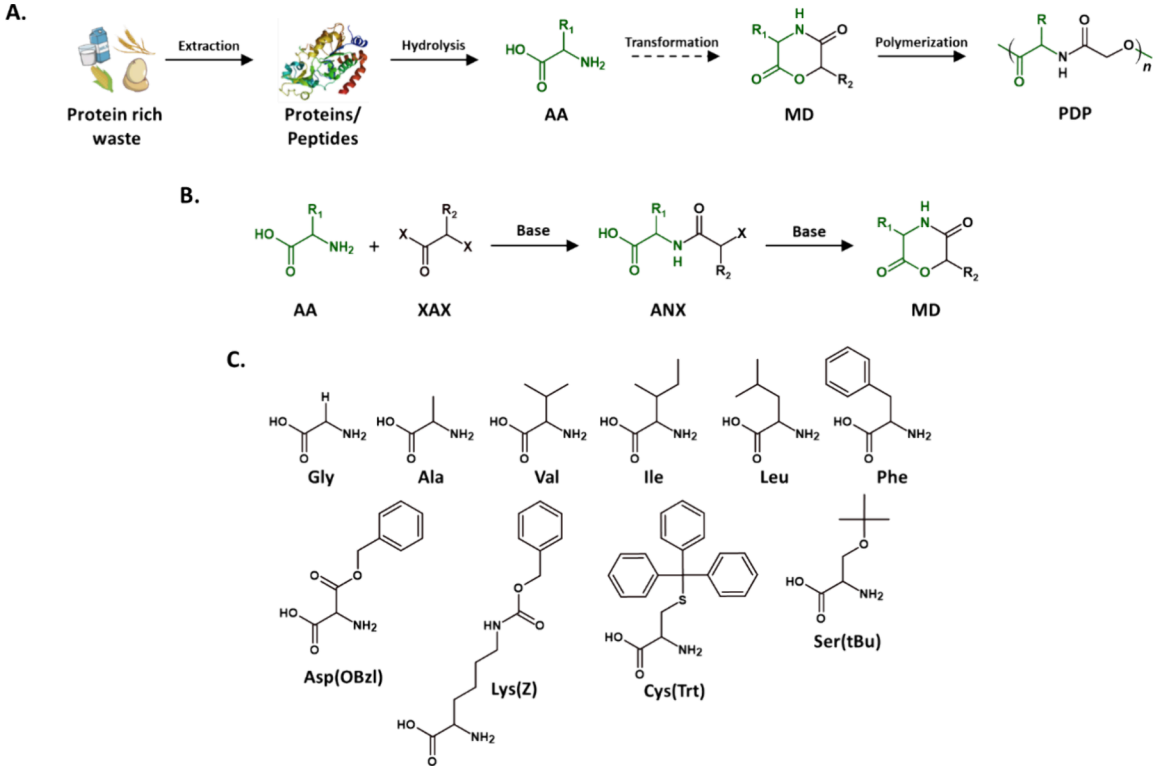

Figure 1. Route to biosourced polydepsipeptides (PDPs) from protein-rich waste (A), synthesis of an MD using an AA (B), and AAs used in this study (C).

MDs can be found naturally in small quantities in flowers,[21] mushrooms,[22,23] sea cucumbers,[24] bacteria,[25] and algae[26] and in earth samples.[27] The production of MDs using AAs as the raw material has been performed via many different methods using various synthesis paths, conditions, and catalysts.[19,28−32] The most common and easiest route for MD production consists of a two-step process wherein the AA is first reacted with an α-halogenated acyl halide to form a *N*-(α-haloacyl)-α-amino acid (ANX) (Figure 1B).[33] This reaction is usually performed in solution with a base as to favor the reaction of the halogenated reagent with the amine group over the acid group. For this reaction, triethylamine is generally favored when working in a homogeneous phase,[18,34−36] while sodium hydroxide is favored when working in Schotten−Baumann biphasic conditions.[37] As to avoid condensation reactions of ANXs, this reaction is typically performed at temperatures ranging between −5 °C and room temperature. Furthermore, the nature of the halide has little influence on the overall yield of the reaction. The second step of the MD synthesis, the cyclization of the ANXs, can be performed in solution or in bulk. In the absence of a solvent, the ANXs can undergo cyclization at high temperatures (120 to 200 °C) under vacuum (10^{-5} mbar); however, substantial product loss occurs due to detrimental condensation reactions.[15,16,38,39] Additionally, MDs derived from AAs carrying protecting groups cannot be obtained via this method, as the high temperatures induce the thermolysis of the protecting groups. Only dimethylformamide (DMF) is reported as the solvent for the solution-based cyclization of ANXs wherein highly dilute conditions are required as to favor the intramolecular reaction over detrimental intermolecular reactions. The solution-based cyclization is typically performed with temperatures ranging from 60 to 110 °C, in the presence of an amine or carbonate-type base. As the synthesis of MDs is well-documented, a wide variety of different conditions are employed and generally vary depending on the nature of the AA used.

In the present study, our objective is to develop a method of valorizing hydrophobic AAs, without the need for prior isolation of the individual AA. Herein, we report an approach for MD synthesis that is common to a series of naturally occurring hydrophobic AAs including Leu, Val, Ile, and Phe as well as using the functional AAs Asp(OBzl), Lys(Z), and Ser(tBu) (Figure 1C). To this end, we screened different conditions as to achieve the most efficient and highest-purity yielding conditions. Next, we applied the optimized procedure to a blend of hydrophobic AAs, thus producing a mixture of MDs. Finally, we probed the possibility of using such MD blends to create polymer materials via ROP as to validate this methodology for valorizing protein hydrolysates without extensive amino acid separation.

2. MATERIALS AND METHODS

2.1. Materials. Alanine (Ala), leucine (Leu), valine (Val), phenylalanine (Phe), isoleucine (Ile), L-aspartic acid β-benzyl ester (Asp(OBzl)), *tert*-butyl-serine (Ser(tBu)), N_α-(carbobenzyloxy) lysine (Lys(Z)), and *S*-trityl cysteine (Cys(Trt)) used herein were of L configuration. These amino acids (>98%) were purchased from Bachem or Sigma-Aldrich and used as received. Chloroacetyl chloride (>98%), bromoacetyl bromide (>98%), bromoacetyl chloride (>98%), and triethylamine (>99.5%) were procured from Sigma-Aldrich, used as received, and stored under nitrogen. The bases NaOH, Na_2CO_3, $NaHCO_3$, and TEA were obtained as reagent grade from different vendors. CTAB (98%), $MgSO_4$ (Redi-Dri), SiO_2 (60 Å, column chromatography grade), and Al_2O_3 (58 Å,

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Brockmann I grade) were procured from Sigma-Aldrich and used as received.

2.2. Instruments. The FTIR spectra were recorded on a PerkinElmer Spectrum 100 spectrometer by using the attenuated total reflectance technique with a ZnSe crystal. Samples were analyzed with 6 scans between 650 and 4000 cm^{-1} with a resolution of 4 cm^{-1}.

^{1}H and ^{13}C spectra were recorded on either a Bruker Avance III HD (400 MHz) equipped with a 9 T magnet and a BBI probe or a Bruker Avance equipped with a 9 T magnet and a BBFO probe. Chemical compounds were dissolved in appropriate deuterated solvents at concentrations of approximately 10 mg mL^{-1}. Chemical shifts (δ) are reported in parts per million (ppm) with TMS as an internal standard. Heteronuclear single quantum coherence NMR spectroscopy was carried out on a 600 MHz Bruker Avance III HD spectrometer equipped with a CryoProbe Prodigy.

Size exclusion chromatography (SEC) was performed at 30 °C in THF at a flow rate of 1.0 mL min^{-1}. Two 5 μm PLgel Mixed D columns were used with a 5 μm PLgel guard column. A Varian 390-LC refractive index detector (RID) was used as the detector. Universal calibration was done using Agilent Technologies EasiVial polystyrene (PS) standards, using the intrinsic viscosities issued by the supplier. Concentrations of approximately 5 mg mL^{-1} were used for the polymer samples.

The elemental compositions of the compounds produced herein were determined with an Elementar Vario Micro Cube. For carbon, hydrogen, and nitrogen, samples were heated to 1150 °C in tin pans, and the resulting gases were reduced to N_2, CO_2, H_2O, and SO_2 over catalytic copper at 850 °C. These gases were then separated in an Elementar TPD column using helium as an eluant then detected and quantified with a katharometer. For oxygen, samples were heated to 1050 °C in silver pans under a helium atmosphere. The resulting gases were converted to CO by pyrolysis and then separated and detected as described previously.

Differential scanning calorimetry (DSC) analysis of polymers was performed by using a Netzsch DSC200F3 calorimeter. Constant calibration was performed with biphenyl, indium, bismuth, zinc, and cesium chloride standards. Nitrogen was used as the purge gas. Approximately 10 mg of the polymer sample was placed in pierced aluminum pans and heated to 180 °C then cooled to 20 °C with a rate of 5 °C min^{-1} with the intention of erasing the thermal history. The thermal properties were then recorded with a heating rate of 10 °C min^{-1} between 20 and 180 °C. The thermal transition temperature was determined from the second heating run.

2.3. Synthesis of LeuCl. *2.3.1. Procedure A.* The following procedure corresponds to the synthesis of LeuCl using Leu, ClACl, and NaOH. For the synthesis of the other ANXs, all parameters and weights were identical except for the AA whose weight was adapted for equivalent molarity (68 mmol).

A 200 mL round-bottom flask was charged with 9 g of Leu (68 mmol, 1 equiv) and 80 mL of a sodium hydroxide solution (2N). The reaction medium was then cooled to 0 °C with an ice bath, and 8 g of ClACl (70 mmol, 1 equiv) in 80 mL of a 50:50 (vol) solution of diethyl ether (EtOEt) and dioxane was added under vigorous agitation over a period of 20 min. Once the addition of ClACl was complete, the reaction was stirred at room temperature for another 20 min. Then, HCl (3 N) was added dropwise until the pH of the reaction media reached 1. The organic phase was then extracted three times with 100 mL of hexane and dried over $MgSO_4$. Finally, the solvents were removed in vacuo, and the resulting yellow solid was recrystallized in a minimal amount of hot ethyl acetate (80 °C) (10.7 g, yield = 75%).

2.3.2. Procedure B. The following procedure corresponds to the synthesis of LeuCl using Leu, ClACl, and Na_2CO_3. For the synthesis of the other ANXs, all parameters and weights were identical, except for AA whose weight was adapted for equivalent molarity (68 mmol).

A 500 mL round-bottom flask was charged with 9 g of Leu (68 mmol, 1 equiv), 7.5 g of Na_2CO_3 (70 mmol, 1 equiv), and 200 mL of THF. Under vigorous stirring, 8 g of ClACl (70 mmol, 1equiv) dissolved in 20 mL of THF was added dropwise to the solution at room temperature over a period of 20 min. The reaction medium was then stirred for a further 5 h. The reaction mixture was then filtered, and the white solid was kept for a future reaction. The filtrate was washed with 50 mL of water; then, the organic phase was extracted three times with ethyl acetate and dried over $MgSO_4$. The solvents were then removed in vacuo, and the resulting yellow solid was recrystallized in a minimal amount of hot ethyl acetate (80 °C) yielding white crystals (9.7 g, yield = 68%).

2.4. Synthesis of MD(Leu). *2.4.1. Procedure C.* The following procedure was used for the synthesis of MD(Leu) using LeuCl. For the synthesis of the other MDs, all parameters and weights were identical except for the ANX whose weight was adapted to maintain 25 mmol of ANX.

Five g of LeuCl (25 mmol equiv) was dissolved in 80 mL of DMF and added dropwise into a solution containing 6.5 g of $NaHCO_3$ (77 mmol) and 720 mL of DMF for 8 h at 60 °C under vigorous stirring. Then, the solution was stirred for another 24 h at 60 °C. The solution was cooled to 0 °C, and the solid was removed by filtration. After the removal of DMF by distillation under vacuum at 40 °C, the residue was washed with 200 mL of ethyl acetate (AE) and 100 mL of water; then, the organic phase was separated and washed with 200 mL of water and dried over $MgSO_4$. The AE was then removed under vacuum, and the resulting solid was recrystallized in a minimal amount of AE at 80 °C yielding white crystals (2.14g, yield = 52%).

2.5. Polymerization of MD Blends. An oven-dried 5 mL pear-shaped flask was loaded with 250 mg of the MD mix (nMD(Leu) = 0.50 mmol, nMD(Ile) = 0.36 mmol, nMD-(Phe) = 0.29 mmol, and nMD(Val) = 0.26 mmol, 25 equiv MD) and placed at 50 °C under vacuum for 2 h. The flask was then back flushed with nitrogen, and 50 μL of a 0.12 g mL^{-1} solution of BnOH (0.056 mmol, 1 equiv) in anhydrous dioxolane and 50 μL of a 0.23 g mL^{-1} solution of $Sn(Oct)_2$ (0.028 mmol, 0.5 equiv) equally in anhydrous dioxolane were added to the flask. The solvent was then removed by 3 nitrogen/vacuum cycles. The flask was then stirred at 110 °C for 24 h, after which the vessel was cooled to room temperature. The polymer was dissolved in 1,3-dioxolane and precipitated in cold diethyl ether to yield the copolymer as a white powder (104 mg, yield = 42%).

3. RESULTS AND DISCUSSION

3.1. Optimization of Morpholine-2,5-dione Synthesis. In the first part of this study, we optimized the synthesis of leucine-derived MD (MD(Leu)) in order to achieve the highest possible yield while employing the simplest reaction and purification procedures as possible. Leucine was chosen as a starting point as we have previously mastered its

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Scheme 1. Synthesis of Leu-Derived MD(Leu) with a LeuCl Intermediary

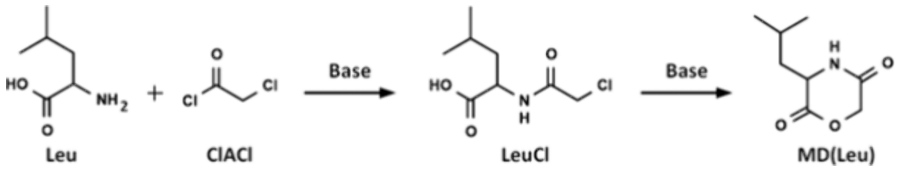

Table 1. Results of the Synthesis of LeuCl from Leu Using an Aqueous Solution of NaOH[a]

experiment	reagent	solvent	time (min)	purification	yield (%)	remark
E1	ClACl	EtOEt	420	acidification > filtration > extraction with hexane > drying	73	impurities in the product
E2	ClACl	EtOEt	420	acidification > filtration > extraction with hexane > recrystallization	49	LeuCl in the filtrate
E3	ClACl	EtOEt	420	acidification > extraction with EA > drying > recrystallization	67	
E4	ClACl	EtOEt	20	acidification > filtration > extraction with hexane > recrystallization	64	
E5	ClACl	dioxane: EtOEt	20	acidification > filtration > extraction with hexane > recrystallization	75	
E6	BrABr	dioxane:EtOEt	20	acidification > extraction with EA > drying	72	impurities in the product
E7	ClABr	dioxane:EtOEt	20	acidification > extraction with EA > drying	70	impurities in the product

[a]Reactions performed at 0 °C.

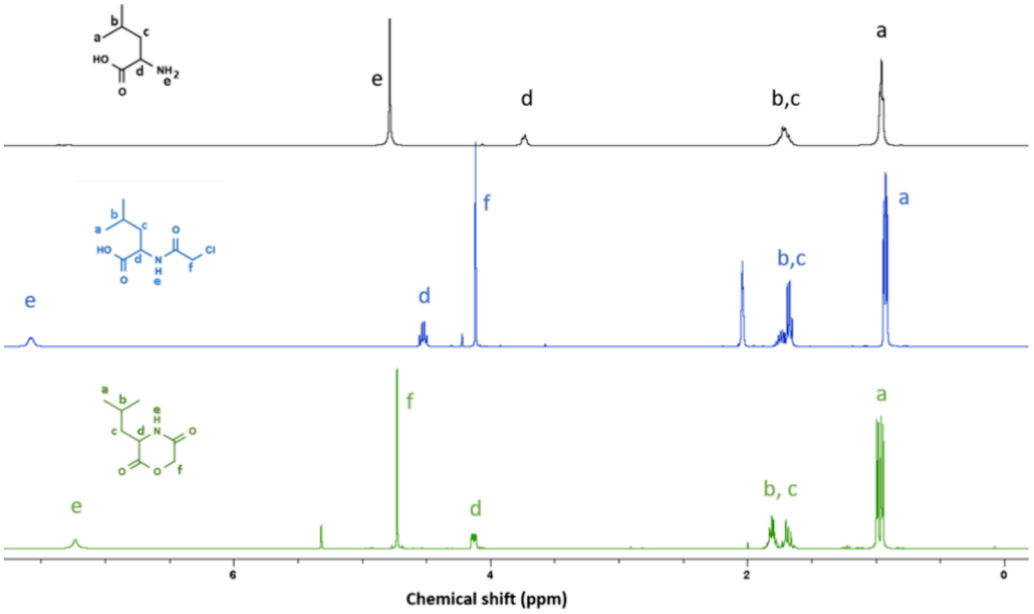

Figure 2. ^{1}H NMR spectra of Leu (black), LeuCl (blue), and MD(Leu) (green). ^{1}H NMR of Leu was measured in deuterated acetone, and ^{1}H NMR of LeuCl and MD(Leu) was measured in $CDCl_3$. 400 MHz.

production.[20] The route using an ANX intermediate was chosen. The first step of this reaction involves the reaction of Leu with chloroacetyl chloride (ClACl) yielding 2-(2-chloroacetamido)-4-methylpentanoic acid (LeuCl) with a base (Scheme 1). For this step, two opposite approaches were tested: the first uses a strong base, and the second uses a weak base.

3.1.1. Synthesis of LeuCl with NaOH. The production of LeuCl under Schotten−Baumann conditions, wherein sodium hydroxide is used as the base with two solvents of opposite polarity to form a biphasic system, was the first explored. Several conditions were tested for the optimization of this step (Table 1).

In E1, ClACl was added dropwise to Leu in stoichiometric quantities within a solvent system composed of diethyl ether and a solution of NaOH (pH 12) at 0 °C (E1, Table 1).[15] After 7 h of reaction, LeuCl was precipitated via acidification of the reaction medium and then filtered and dried, giving a mass yield of 73%. Further recrystallization of as-obtained LeuCl in ethyl acetate (EA) provided a pure product, as attested by elemental analysis, with a final yield of 49% (E2, Table 1). However, a significant amount of LeuCl was found in the filtrate. The structure of LeuCl was confirmed by NMR (^{1}H and HSQC) (Figure 2, Figure S1, and Table S1) and FTIR (Figure 3). Indeed, the NMR spectrum of the product displays characteristic signals of Leu ("a" to "e") in addition to a new peak ("f") at 4.1 ppm that is characteristic of the added acetamide chloride moiety. Furthermore, a downfield shift can be observed for the signals assigned to the proton ("d") carried by the asymmetric carbon and the proton ("e") carried by the nitrogen, indicative of changes in their chemical environment. Moreover, the relative areas of all of these peaks correspond well to those expected for LeuCl. HSQC-NMR analysis further confirmed the structure of the final product (see Figure S1). The FTIR spectrum of LeuCl (Figure 3) presents vibration bands characteristic of an amide function (N−H elongation at

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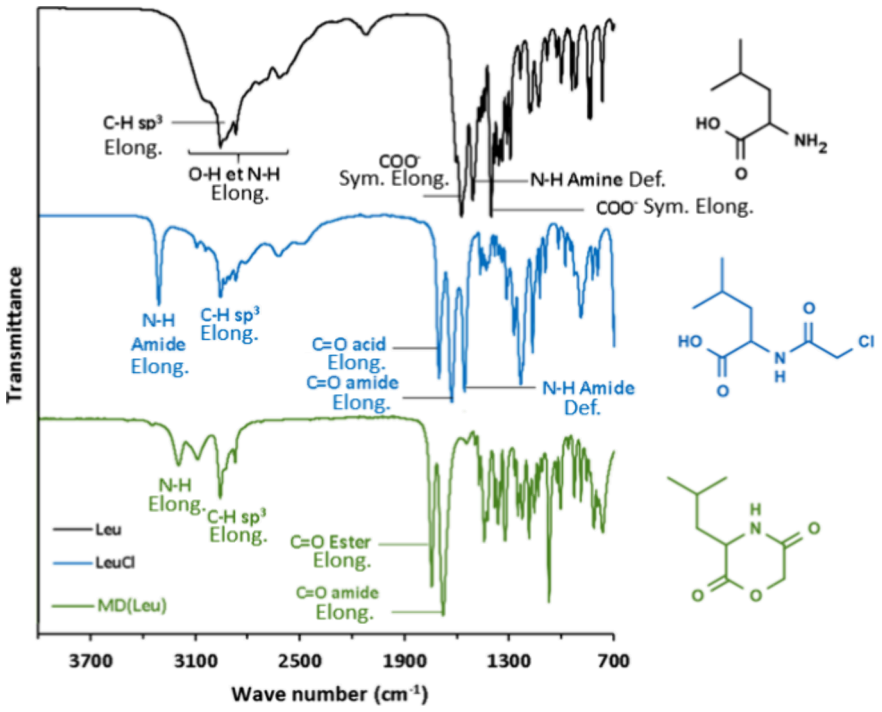

Figure 3. FTIR spectra of Leu (black), LeuCl (blue), and MD(Leu) (green).

Table 2. Results of the Synthesis of LeuCl Using Leu, ClACl, and a Weak Base

experiment	base	solvent	T (°C) and time	purification	yield (%)	remarks
E8	Na_2CO_3 + 1% CTAB	THF + few drops of water	20 °C for 5 h and then 70 °C for 8 h	filtration > washing > drying	0	polycondensation
E9	Na_2CO_3 + 1% CTAB	THF + few drops of water	20 °C for 5 h	filtration > washing > drying	83*	impurities and traces of CTAB
E10	Na_2CO_3 + 1% CTAB	THF + few drops of water	20 °C for 5 h	filtration > washing > drying > recrystallization	76*	traces of CTAB
E11	Na_2CO_3	THF	20 °C for 5 h	filtration > washing > drying > recrystallization	68	

3312 cm^{-1}, C═O elongation at 1631 cm^{-1}, and N−H deformation at 1558 cm^{-1}), an acid function (C═O elongation at 1704 cm^{-1}), and the alkyl group (C−H stretching at 2950 cm^{-1}). The experimental elemental composition was found to be close to the theoretical composition of LeuCl (Table S2). Finally, the melting point of LeuCl (138 °C) was measured by differential scanning calorimetry (DSC) and proved much lower than that of leucine (286 °C), which could be indicative of less hydrogen bonding. The formation of the target LeuCl was thus confirmed.

In order to improve the yield of E2, the reaction and purification conditions were further explored. First, the composition of the filtrate from E2 was investigated by ^1H NMR spectroscopy, which confirmed the presence of a non-negligible quantity of LeuCl, as observed in other studies.[39−42] Consequently, the LeuCl within the filtrate was extracted with EA, dried, and recrystallized, allowing improvement of the yield by almost 20% (total yield = 67%) (E3, Table 1). Second, the time of the reaction was reduced as performed in some studies.[37,39−43] Thus, the reaction was conducted over a shorter time frame of 20 min producing LeuCl with a yield of 64% (E4, Table 1). Next, the influence of the solvent was studied. Switching from pure diethyl ether as an organic phase to a 50:50 v/v mixture of dioxane:diethyl ether allowed us to improve the yield of the reaction to 75% (E5, Table 1). Finally, when using brominated intermediaries such as bromoacetyl bromide (BrABr) and chloroacetyl bromide (ClABr) in place of ClACl (E6 and E7, respectively, Table 1), the yield before recrystallization was found to be lower than the yield after recrystallization when using ClACl (E5, Table 1). These results demonstrate that ClACl is more efficient for this reaction than acyl bromides. The procedure for generating LeuCl with NaOH using the optimal conditions determined above (E5, Table 1) is described in the Materials and Methods section and is named procedure A.

3.1.2. Synthesis of LeuCl with Na_2CO_3. A softer approach for the synthesis of LeuCl was also investigated by using less harsh conditions and more simple procedures. Jursic and Neumann have demonstrated the amidation of hydrophobic amino acids with acid chlorides using Na_2CO_3 and cetyltrimethylammonium bromide (CTAB) as the phase transfer agent.[44] The conditions employed in that study were reproduced for the synthesis of LeuCl from Leu and ClACl (E8, Table 2) using THF as the solvent in addition to a few drops of water. Initially, the reaction mixture was stirred at room temperature for 4 h and then left to reflux for 8 h. After filtration of the resulting mixture, the organic phase was extracted with EA and dried over $MgSO_4$, and the solvents were removed (in vacuo). Unfortunately, no peaks correspond-

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Table 3. Results of the Synthesis of MD(Leu) from LeuCl

experiment	base	solvent	T (°C)	t (h)	purification	yield (%)	remark
E12	$NaHCO_3$	DMF	60	24	drying > washing > drying > recrystallization × 2	55	
E13	TEA	DMF	60	24	drying > washing > drying > recrystallization × 2	41*	impurities
E14	$NaHCO_3$	DMF	25	24	drying > washing > drying > recrystallization × 2	0*	raw product = LeuCl
E15	$NaHCO_3$	acetone/DMC/DMSO, *n*-BuOH/ACN/THF	60	24	drying > washing > drying > recrystallization × 2	0*	raw product = LeuCl
E16	$NaHCO_3$	DMF	60	24	drying > washing > drying > recrystallization × 2 > evaporation of DMF at 40 °C	0*	ring opening
E17	$NaHCO_3$	DMF	60	24	filtration > evaporation of DMF at 40 °C > washing > drying > recrystallization × 2	52	

ing to LeuCl were observed in the ^{1}H NMR spectrum of the resulting solid (see Figures S2 and S3 and Table S1). We believe that an abundance of side reactions, such as condensation, occurred due to the higher temperatures involved. Consequently, E8 was reproduced at a lower temperature (20 °C) giving a final product with a significant yield of 83% and without side reactions (E9, Table 2). However, small amounts of CTAB remained in the resulting product that could not be removed even after recrystallization (E10, Table 2). Therefore, E10 was reproduced without CTAB, leading to the production of pure LeuCl with a satisfactory yield of 68% (E11, Table 2). The procedure for generating LeuCl with Na_2CO_3 using the optimal conditions determined above (E11) is described in the Materials and Methods section and is named procedure B.

3.1.3. Optimization of the Cyclization of LeuCl. The production of MD(Leu) via the intramolecular cyclization of LeuCl was optimized using typical reaction conditions from the literature as a starting point.[45,46] The reaction was thus first carried out at 60 °C for 24 h using $NaHCO_3$ as a base and DMF as the solvent (E12, Table 3). In those conditions, an MD yield of 55% was achieved after two recrystallizations in EA. As our intent is the polymerization of MD(Leu), the purity of the final product is important; thus, ^{1}H NMR, ^{13}C NMR, FTIR spectroscopy, and elemental analysis were performed. The ^{1}H NMR spectrum of the resulting product presents peaks characteristic of the isobutyl protons ("a", "b", and "c"), which are identical to those for LeuCl, in addition to peaks characteristic of the methine ("d") and methylene ("f") protons on the ring (Figure 2). Those two protons ("d" and "f") undergo strong deshielding and shielding (respectively) when compared to those of LeuCl. Furthermore, the relative areas of all the peaks in the ^{1}H NMR spectrum of MD(Leu) correspond to the number of protons to which they have been assigned (Figure 2). The ^{13}C NMR spectrum of MD(Leu) in $CDCl_3$ (Figure S4) displays two peaks at 167.3 and 166.4 ppm corresponding to the carbonyls of the ester and amide moieties, respectively. The carbon peaks at positions 3 and 6 on the ring ("d" and "f") have chemical shifts at 51.9 and 67.5 ppm, respectively. Finally, four peaks at 41.6, 24.3, 23.0, and 21.4 ppm can be assigned to the 4 carbons of the isobutyl group. Importantly, these spectra do not indicate the presence of any impurities and are identical to those reported in the literature.[18,47]

Good obtention of MD(Leu) was also confirmed by FTIR analysis. Indeed, both FTIR spectra of MD(Leu) and LeuCl (Figure 3) display the two absorption bands at 1679 and 1742 cm^{-1} corresponding to the carbonyls of the amide and ester, respectively. In addition, there is a strong attenuation of the characteristic absorption bands of the N−H bond between the FTIR spectrum of LeuCl and that of MD(Leu), whether in elongation (1600 cm^{-1}) or strain vibration (3250 cm^{-1}) modes, as it is usually observed with cyclic amides when compared to linear amides.[48−50] Finally, the characteristic absorption bands of the isobutyl C−H bonds are visible at 2960 cm^{-1}. This FTIR spectrum is identical to a previous report.[18]

The elemental analysis of the recrystallized product revealed that the mass composition of the purified product corresponds to those estimated for MD(Leu) (see Table S3), attesting to the product purity.

Applying another base as triethylamine (TEA) for the cyclization reaction (E13, Table 3) did not improve the yield (41%), indicating that $NaHCO_3$ is more suited for this reaction. The cyclization was then attempted at a lower temperature (25 °C) as this reaction has a relatively high energy consumption; however, no MD(Leu) was detected (E14, Table 3). Next, we attempted exchanging DMF for a less hazardous solvent with a lower environmental impact. Various solvents were examined including acetone, dimethyl carbonate (DMC), *n*-butanol (*n*-BuOH), dimethyl sulfoxide (DMSO), acetonitrile (ACN), and THF; however, no MD(Leu) was detected in any of those solvents (E15, Table 3).

We then went on to optimize and simplify the workup procedure. A particularly laborious step of this protocol is the evaporation of the DMF under vacuum from the reaction medium at room temperature. When increasing the vacuum distillation temperature to 40 °C, the concentrated MD(Leu) undergoes ring-opening reactions in the presence of $NaHCO_3$ (E16, Table 3). Since $NaHCO_3$ is sparsely soluble in DMF, this salt can be efficiently removed from the reaction medium by cooling the mixture to 0 °C. In this manner, it was found that the vacuum distillation of DMF can be quickly carried out at 40 °C with no secondary reactions taking place, effectively reducing the workup time by 4 to 5 h. Once washed and recrystallized, a yield of 52% was achieved (E17, Table 3). The procedure for generating MD(Leu) using the simplified purification process (E17, Table 3) is described in the Materials and Methods section and is named procedure C.

3.1.4. Application of the Optimized Procedures to a Range of AAs. Using the optimized procedures of MD(Leu) synthesis from Leu determined above, we attempted to synthesize a range of different MDs. For this, both hydrophobic AAs carrying no functional groups (Ile, Phe, Val, Gly, and Ala) and AAs carrying protected functional groups (Asp(OBzl), Lys(Z), Cys(Trt), and Ser(tBu)) were tested. Although the latter protected AAs are not obtained from protein hydrolysates, the introduction of such functions could

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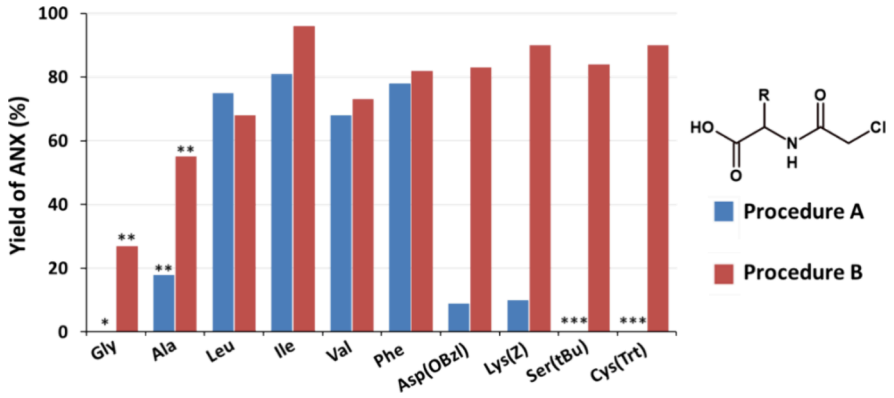

Figure 4. Yield of the synthesis of ANXs from different starting AAs using procedure A (blue) or procedure B (red).*: No ANX detected; **: considerable impurities in the final product; ***: the reaction was not performed.

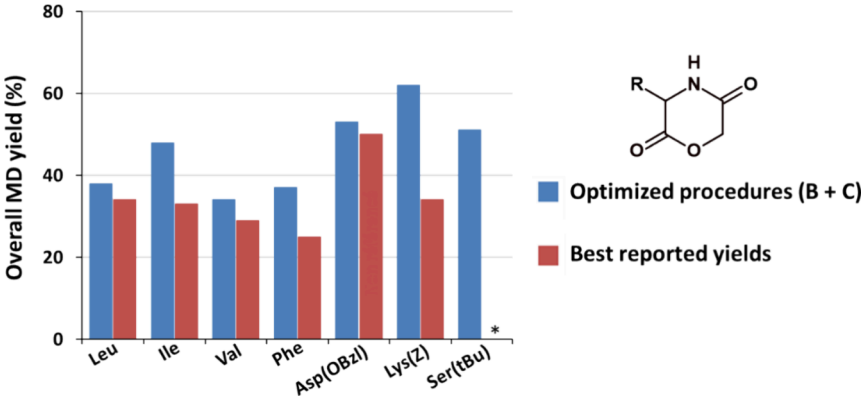

Figure 5. Global yields of the synthesis of the different MDs using procedures B and C compared to the global yields found in the literature for MD(Leu),[18] MD(Ile),[51] MD(Val),[52] MD(Phe),[53] MD[Asp(OBzl)],[42] and MD[Lys(Z)].[41]

be interesting for the applications of copolydepsipeptides in the biomedical field. For each AA, both procedures A and B were employed for producing the ANX intermediary, and procedure C was used for the cyclization of the ANXs. The resulting yields from procedures A and B are compared in Figure 4. In the case of Val, Ile, and Phe (AAs with hydrophobic character similar to that of Leu), their corresponding ANXs were obtained with good yields using both procedures. Minor differences in the ANX yields are observed with aliphatic AAs Leu, Ile, and Val and aromatic AA Phe, which can be explained by their slight difference of solubility in ethyl acetate, which is employed during the purification step. A significant difference between these two procedures appeared with Asp(OBzl) and Lys(Z). Indeed, modest yields of Asp(OBzl)Cl and Lys(Z)Cl were obtained with procedure A, whereas very good yields were achieved with procedure B. Furthermore, Cys(Trt)Cl and Ser(tBu)Cl were accessed in sufficient yields thanks to the procedure B. Since procedure A uses a two-phase medium and procedure B uses an organic medium only and since the protected amino acids exhibit a high hydrophobic character, the yields are higher when using procedure B than using procedure A. When applying the optimized procedure B to glycine and alanine, the corresponding ANX intermediaries were successfully obtained; however, a considerable amount of impurities persisted that could not be eliminated after two recrystallizations in EA. We believe that this can be attributed to the more pronounced hydrophilic nature of these compounds compared to the other AAs for which the purification was successful (Leu, Ile, and Phe). For this reason, glycine and alanine were not used for the rest of the study. Nonetheless, optimization of the composition and nature of the solvents used for the trituration and the recrystallization could potentially resolve this issue. In view of these results, it can be concluded that the production of ANXs is more efficient and reliable with procedure B (with Na_2CO_3) than with procedure A (with NaOH) and is applicable to many different AAs. The structure of each ANXs has been confirmed by ^{1}H NMR and their purity by elemental analysis (see Figure S3 and Tables S1 and S2). For the following part of this study, the ANXs obtained via procedure B were employed.

3.1.5. Cyclization of ANXs. The cyclization of the different ANXs obtained above was carried out using procedure C employed for the synthesis of MD(Leu). All MDs (except for the MD derived from Cys(Trt)) were successfully synthesized from their corresponding ANXs in relatively good yields: MD(Leu), 55%; MD(Ile), 50%; MD(Val), 46%; MD(Phe), 45%; MD[Asp(OBzl)], 64%; MD[Lys(Z)], 69%; MD[Ser(tBu)], 61%. It thus appears that more sterically hindered ANXs result in higher yields of MDs, which may be attributed to a favored intramolecular cyclization as compared to the intermolecular reaction. In addition, the problematic cyclization of cysteine-based ANX has previously been observed and can be explained by the deprotection of the thiol group leading to intermolecular reactions or oxidation reactions.[34,37] The structure and purity of each MD were characterized by ^{1}H and ^{13}C NMR spectroscopy, FTIR spectroscopy, elemental analysis, and melting point analysis (see Figures S5 to S7 and Tables S3 to S5). The combined yields of produced MDs

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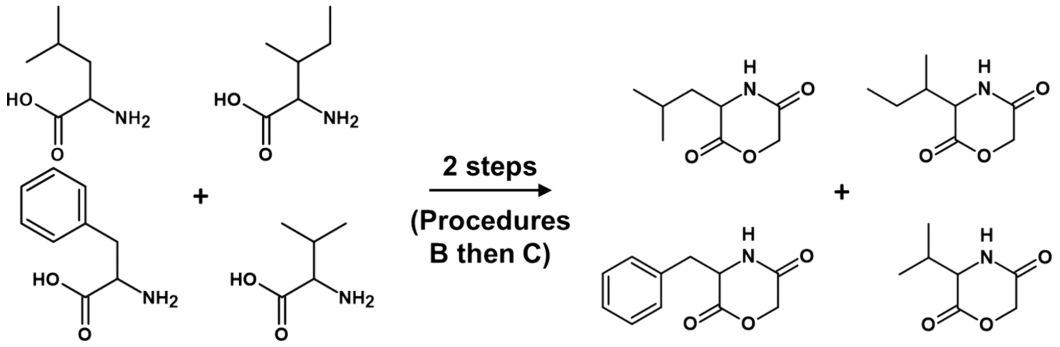
Scheme 2. Synthesis of a Blend of MD(Leu), MD(Ile), MD(Phe), and MD(Val) Using an Equimolar Mixture of Leu, Ile, Phe, and Val



from the AAs following procedures B and C are represented in Figure 5. It should be noted that the overall yields of the MDs obtained herein surpass those reported in the literature. Importantly, exactly the same procedure was used for each of these MDs, and products of excellent purity were obtained.

3.2. Synthesis of MDs from a Blend of AAs. Next, we probed the possibility of applying the above procedures (B and C) to cogenerate a mixture of MDs from a blend of hydrophobic AAs. For this test, the naturally occurring AAs Val, Ile, Leu, and Phe were used in equimolar amounts (Scheme 2).

The reaction of this AA blend with chloroacetyl chloride (ClACl) using the conditions defined in procedure B yielded a mixture of ANXs that could be effectively recrystallized in EA. Analysis by ^{1}H NMR spectroscopy of the as-obtained solid allowed the quantification of each individual ANX within this product (Figure S8). These proportions (Table 4) remained relatively close to those of the initial fraction of AAs with X_{LeuCl} = 0.26, X_{ValCl} = 0.20, X_{IleCl} = 0.28, and X_{PheCl} = 0.26. These ANXs were then cyclized using the procedure C, yielding a blend of corresponding MDs that could be effectively purified via recrystallization in EA. Similarly, ^{1}H NMR spectroscopy (Figure S9) revealed that the proportions of MDs within this product were $X_{MD(Leu)}$ = 0.35, $X_{MD(Ile)}$ = 0.26, $X_{MD(Phe)}$ = 0.21, and $X_{MD(Val)}$ = 0.18. The overall yield of this synthesis was 35%, which is slightly less than those of MD(Leu), MD(Phe), and MD(Ile) synthesized individually.

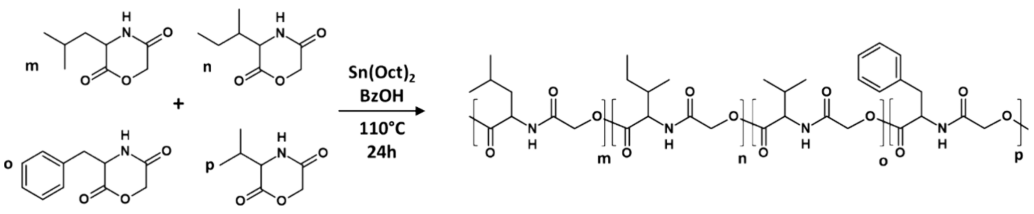
Table 4. Compositions of the Initial AA Mixture and the Resulting ANX and MD Mixtures

AA	X^{AA}_{mol}	X^{ANX}_{mol}	X^{MD}_{mol}	M_{MD} (g mol^{-1})	X^{MD}_{mass}
Leu	0.25	0.26	0.35	171.20	0.341
Ile	0.25	0.28	0.26	171.20	0.253
Phe	0.25	0.26	0.21	205.21	0.245
Val	0.25	0.20	0.18	157.17	0.161

3.3. Exploratory Polymerization of MD Blends. As to prove the concept of creating copolydepsipeptides from a blend of hydrophobic AAs, we attempted the copolymerization of the produced MD blend containing the 4 morpholine-2,5-diones MD(Leu), MD(Phe), MD(Ile), and MD(val). To this end, a typical ring-opening polymerization catalytic system composed of tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) and benzyl alcohol (BzOH) was employed. The polymerization was carried out under bulk conditions at 110 °C for 24 h using the following monomer/initiator/catalyst ratio MD/BzOH/$Sn(Oct)_2$ = 50/2/1 (Scheme 3). If all MDs are fully converted to the corresponding copolydepsipeptide, this should provide a polymer with a molecular weight of 4.4 kg mol^{-1} (see Figure S10).

After 24 h of reaction, the reaction mixture was dissolved in 1,4-dioxolane and precipitated into cold diethyl ether. As can be observed in Figure S11, the ^{1}H NMR spectrum of the precipitated copolymer displays broad peaks corresponding to each of the protons found on all four of the MD repeating units. Further analysis of the copolymer by ordered diffusion spectroscopy (NMR DOSY) indicates that the resulting polymer exhibits only a single diffusion coefficient, as shown in Figure S12. Given that the protons in each MD unit diffuse at the same rate and not at different diffusion coefficients, it can be concluded that each copolymer chain contains all four MDs. The thermogram presented in Figure S13 also confirms the formation of a copolymer, as only one glass transition temperature (T_g) is observed. The size exclusion chromatogram of the resulting material revealed the formation of a polymer presenting a unimodal distribution of molecular weight with a dispersity of 2.0 and an average molecular weight of 2.2 kg mol^{-1} (vs PS standards) (Figure S14). This experiment demonstrates that the MD mixture produced with the process developed herein can successfully undergo

Scheme 3. Copolymerization of a Blend of MD(Leu), MD(ILe), MD(Phe), and MD(Val) Using $Sn(Oct)_2$ as a Catalyst and BzOH as an Initiator



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polymerization to form copolydepsipeptide materials. Optimizing the reaction conditions or using another type of catalytic system such as organocatalysts could yield polymers with higher molecular weights and precise structures.[17]

4. CONCLUSIONS

An optimized method for producing morpholine-2,5-diones was determined using simple procedures while simultaneously minimizing hazardous chemicals. Importantly, the procedure that we developed is in practice identical for an array of different hydrophobic amino acids: Leu, Val, Ile, and Phe as well as for side group-protected AAs based on Lys, Asp, and Ser. This optimized procedure permitted the synthesis of MDs with better yields than those previously reported. Additionally, one new MD was produced: MD[Ser(tBu)]. Owing to the identical nature of that production procedure for different hydrophobic AAs, we successfully demonstrated that mixtures of Leu, Ile, Val, and Phe could be used to generate a blend of MDs (MD(Leu), MD(Ile), and MD(Val)). We then demonstrated that this mixture of MDs can be successfully polymerized to form a copolymer containing repeating units from all four MDs.

The novelty of this approach for creating materials from mixtures of amino acids is a pragmatic first step toward the valorization of protein wastes as their hydrolysates contain many different AAs whose separation is extremely challenging. Approaches to bioderived material production like the one developed herein are essential as raw materials are rarely pure-body chemicals and may sometimes present unique opportunities such as for copolydepsipeptide production. Given the biodegradable and biocompatible nature of polydepsipeptide-type materials, many interesting applications can be imagined for these materials in a broad range of fields. However, this process still displays issues for industrialization, such as the use of a large amount of solvents or the use of anhydrous magnesium sulfate. Consequently, the development of a solvent-free synthesis process for morpholine-2,5-diones is underway in our lab.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02670.

HSQC-NMR spectra of LeuCl (Figure S1); ^{1}H NMR spectrum of the product from E8, Table 2 (Figure S2); proton attributions of ANXs (Figure S3); ^{13}C NMR spectra of MD(Leu) (Figure S4); ^{1}H NMR spectra of MDs at 400 MHz, $CDCl_3$ (Figure S5); carbon attributions of MDs (Figure S6); FTIR spectra of MDs (Figure S7); ^{1}H NMR spectrum (400 MHz) of the mix of LeuCl, ValCl, IleCl, and PheCl in $CDCl_3$ produced from a blend of corresponding AAs and determination of ANX proportions (Figure S8); ^{1}H NMR spectra (400 MHz) of the mixture of MDs (black) obtained using procedures B and C, MD(Leu) (orange), MD(Ile) (purple), MD(Val) (green), and MD(Phe) (blue) in $CDCl_3$ (Figure S9); calculation of the theoretical molecular weight of the copolymer (Figure S10); ^{1}H NMR of the precipitated polymer in $CDCl_3$ (Figure S11); ^{1}H NMR and DOSY-NMR (600 MHz) of the precipitated polymer in $CDCl_3$ (Figure S12); differential scanning calorimetry thermograms of the precipitated polymer (Figure S13); differential molecular weight distribution from size exclusion chromatography measurements of the precipitated polymer (Figure S14); chemical shift of peaks observed in ^{1}H NMR spectra of ANXs (Table S1); elemental compositions of ANXs (Table S2); elemental compositions of MDs (Table S3); chemical shift of observed signals in ^{13}C NMR spectra of MDs (Table S4); melting points of MDs as measured by DSC analysis (Table S5) (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

Notes

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