Synthesis of glycero amino acid-based surfactants. Part 1. Enzymatic preparation of *rac*-1-*O*-(N^{α}-acetyl-L-aminoacyl)glycerol derivatives

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An enzymatic procedure for the selective synthesis of a number of N^{α}-protected amino acid glyceryl esters is presented. Using N^{α}-Boc-arginine as a model substrate, changes in product yield are examined for the following reaction variables: aqueous buffer content, pH, biocatalyst configuration (free or deposited), enzyme concentration and synthetic approach (thermodynamically or kinetically controlled synthesis). The best yields (61–89%) are obtained at 50 °C in glycerol containing a range of aqueous buffer from 0 to 10% (v/v). Readily available industrial proteases and lipases are used as catalysts. The method allowed us to selectively acylate one of the primary hydroxy groups of the glycerol with the carboxylate groups of the amino acid. In the case of aspartic acid and glutamic acid, selective esterification of the α-carboxylate group is achieved. None of the enzymes tested could differentiate between the two enantiotopic hydroxymethyl groups of glycerol, giving diastereoisomeric mixtures in all cases.

Introduction

Amino acid and peptide lipid conjugates constitute an interesting class of speciality surfactants with good surface properties, antimicrobial activity, low potential toxicity and high biodegradability.[1] Moreover, they can be synthesized using selective chemoenzymatic methods from natural renewable sources of raw materials such as amino acids and fatty acids.[2] All these features make them very attractive as dispersants, drug carriers and bactericides in food and pharmaceutical formulations.

Glycero amino acid-based surfactants constitute a novel class of amino acid lipid conjugates, which can be considered analogues of partial glycerides and phospholipids. They consist of one or two aliphatic chains and one polar head, *i.e.* the amino acid, linked together through a glycerol moiety. The resulting structures resemble the acid esters of monoglycerides such as lactic, citric, tartaric and succinic glyceride esters widely used as food emulsifiers.[3] The physicochemical and biological properties of amino acid glyceride conjugates have not been extensively explored yet.[4] From preliminary observations carried out in our lab, these novel compounds combine the advantages of both partial glycerides and lipo-amino acids. For instance, they possess antimicrobial activity, typically from lipo-amino acids, and form lamellar phases and vesicles, characteristic of partial glycerides and phospholipids. Moreover, the possibility of introducing different ionic groups (*i.e.*, selecting the amino acid) increases the swelling properties by promoting the electric repulsion between charged group bilayers. For all these reasons, they constitute a promising family of compounds with a great potential interest in pharmaceutical and food formulations.

One interesting strategy for the synthesis of glycero amino acid-based surfactants consists of the acylation of the hydroxy groups of amino acid and peptide glyceryl ester derivatives.[2a] Amino acid glyceryl esters can be prepared from the corresponding amino acid and either solketal, glycidol or glycerol.[5] Having in mind the use of readily available natural renewable sources of starting materials we chose the simple glycerol. The ester bond between the α-carboxy group of the amino acid and one of the primary hydroxy groups of glycerol can be performed chemically or enzymatically. A specific catalyst such as BF_3 at 50 or 60 °C with an excess of glycerol is the chemical method used.[2a,6] In this case, cumbersome orthogonal protection steps for polyfunctional amino acids are often required. Moreover, acid-sensitive protecting groups, such as *tert*-butoxycarbonyl (Boc), cannot be used due to their instability in the presence of BF_3. Alternatively, enzymatic methodologies using hydrolytic enzymes, notably papain, as catalysts for the ester-bond formation have been employed successfully.[7] However, in this strategy acidic and basic side functions were protected, thereby negating the potential advantages of enzyme selectivity over the chemical method.

The enzymatic preparation of amino acid-based surfactants, of mono and diacylglyceride type, is a current topic of interest in our laboratory. Our pursuit of this goal first led to the synthesis of 1-*O*-(N^{α}-acetylaminoacyl)glycerol derivatives or amino acid $^{\alpha}$COO-glyceryl esters, which are to constitute the polar head of the surfactant. Thus, hydrophilic amino acids with either ionic or non-ionic character, particularly those found in the peptidic sequences of naturally occurring lipopeptides,[8] were selected: arginine, aspartic acid, asparagine, glutamic acid, glutamine and tyrosine. In this paper, we report the enzymatic regioselective synthesis of the aforementioned amino acid glyceryl esters with minimal protection. The amino acids were solely N^{α}-protected with *tert*-butoxycarbonyl (Boc) and, preferentially, with acetyl (Ac). Boc protecting group can be easily removed under acidic conditions. Acetyl, being present in numerous natural structures, allowed the permanent protection of amine functions, preventing their interaction with the acidic side functions and preserving the non-ionic character of the neutral amino acids. Therefore, the goal was to prepare the following amino acid glyceryl esters: Ac-Arg-OGl **2**, Ac-Asp-OGl **3**, Ac-Glu-OGl **4**, Ac-Asn-OGl **5**, Ac-Gln-OGl **6** and Ac-Tyr-OGl **7** including Boc-Arg-OGl·HCl **1**. Special attention

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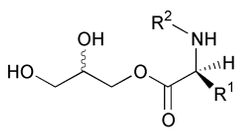
was paid to the purification procedures since a simple solvent extraction cannot be applied in this case. The study was focused on two aspects. First, the influence of water content, biocatalyst type and configuration, enzyme concentration and the pH were systematically investigated. Second, under the best reaction conditions the preparation and purification of the derivatives on a gram scale were performed. The methodology developed provides a general strategy for the synthesis of amino acid and oligopeptides glyceryl esters. These derivatives can also be useful as acyl-donor ester substrates in irreversible lipase- and trypsin-catalysed kinetically controlled peptide synthesis [5–7] and as a strategy to modulate the solubility of single amino acids in pharmaceutical and dermatological formulations.[9]



1 R^1 = -$CH_2CH_2CH_2NHC(NH_2)_2^+Cl^-$, R^2 = Bu^tOCO-

2 R^1 = -$CH_2CH_2CH_2NHC(NH_2)_2^+Cl^-$, R^2 = CH_3CO-

3 R^1 = -CH_2COOH, R^2 = CH_3CO-

4 R^1 = -CH_2CH_2COOH, R^2 = CH_3CO-

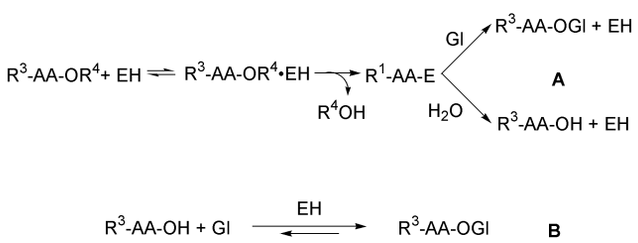
5 R^1 = -CH_2CONH_2, R^2 = CH_3CO-

6 R^1 = -$CH_2CH_2CONH_2$, R^2 = CH_3CO-

7 R^1 = -$CH_2C_6H_4OH$, R^2 = CH_3CO-

Results and discussion

The synthesis of N^{α}-protected amino acid glyceryl esters involves the esterification of one of the primary hydroxy groups of glycerol by the α carboxy group of the amino acid (Scheme 1). Initially, a systematic study was conducted to establish suitable reaction conditions for the selective enzymatic synthesis. For experimental convenience, the synthesis of **1** was used as a model. The ester-bond formation was performed first under kinetically controlled conditions (Scheme 1A) using Boc-Arg-OMe·HCl as acyl donor. Recent results from our lab [2c] and literature data [7] suggested papain as putative catalyst in glycerol, acting as both reactant and solvent, at 50 °C. Preliminary experiments confirmed that the product could be obtained under those conditions using papain on Celite™ as catalyst. A selective monoacylation of one of the primary hydroxy groups of the glycerol was ascertained by ^1H NMR, ^{13}C NMR and mass spectrometric analysis.



R^3 = Boc, Ac; AA: Arg, Asp, Glu, Asn, Gln, Tyr
R^4 = Me, Et; EH: hydrolase; Gl: glycerol

Scheme 1 Kinetically (A) and thermodynamically (B) controlled enzymatic synthesis of 1-*O*-(N^{α}-protected aminoacyl) glycerol derivatives (R^1-AA-OGl): EH protease or lipase (serine or cysteine type in A); R^1-AA-OR^2, acyl-donor ester; R^1-AA-E, acyl-enzyme complex.

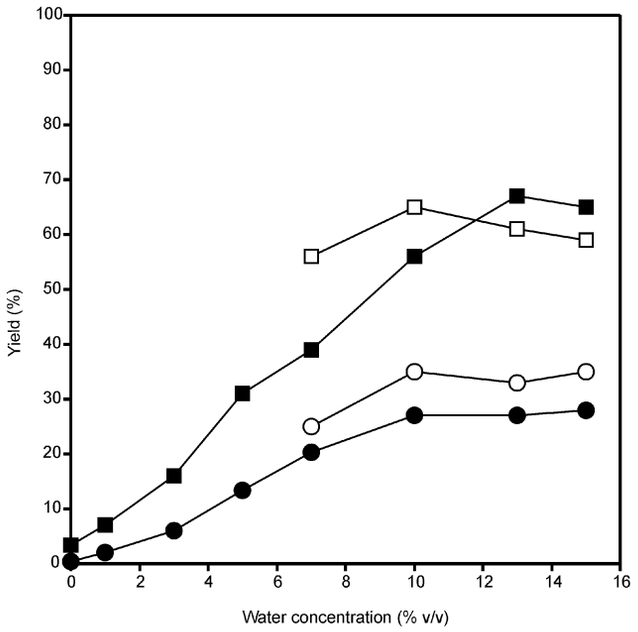


Fig. 1 Kinetically controlled papain-catalysed synthesis of **1**. Influence of aqueous buffer concentration on the product **1** yield (square symbols) and on acyl donor hydrolysis (Boc-Arg-OH·HCl) (circle symbols). Papain deposited onto polyamide (filled symbols) and Celite™ (empty symbols) was used as catalyst. Reaction conditions are described in the Experimental section.

Effect of buffer concentration and support for enzyme deposition

Papain deposited either onto Celite™ or polyamide was placed in glycerol (1 ml), containing the Boc-Arg-OMe·HCl, at 50 °C and the reaction performance was examined as a function of aqueous buffer concentration in the medium. The results obtained, depicted in Fig. 1, show that, working with polyamide, maximum yields (65–67%) were achieved at buffer concentrations ranging between 13% and 15% v/v. Above and below this range the transesterification yields decreased because of an increase of the acyl donor hydrolysis rate (data not show) and either low enzymatic activity or inactivation, respectively. Using Celite™ the system followed a similar trend except that the maximum yields were achieved at 10–13% v/v buffer. A close inspection of the data revealed that the transesterification product : hydrolysed acyl donor ratio for both supports was rather constant (≈2) in the 7–15% v/v buffer range. This suggests that, within this range, the product yield depended on the enzymatic activity rather than on the relative rates of hydrolysis and glycerolysis of the acyl enzyme (Scheme 1A). On this basis, the different optimal buffer content observed with both supports may be explained in terms of enzymatic activity and support aquaphilicity [10] (*i.e.*, the partition of water between the support material and the solvent). Polyamide adsorbed much more water than did Celite™ so that the enzyme is insufficiently hydrated, and thereby less active, requiring more water to be present in the medium.

Influence of the biocatalyst

We have shown that papain from papaya latex can be successfully used in the transesterification of Boc-Arg-OMe·HCl with glycerol. Moreover, we were also interested in investigating the ability of other industrial hydrolases to catalyse this reaction and, in turn, use them for the synthesis of the target amino acid glyceryl esters. To this end, the following lipases and proteases were tested as catalysts: lipases from *Candida antarctica* (Novozym 435™), *Aspergillus niger* (lipase A™), *Pseudomonas cepacia* (lipase PS™), subtilisins from *Bacillus subtilis* (Alcalase™, Protease N), trypsin and bromelain. For comparison purposes, highly purified subtilisin BPN′ was also examined.

Proteases and lipases were deposited onto Celite™, except for Novozym 435™ that was already immobilized. Reactions were performed in glycerol containing 10% (v/v) buffer concentration at 50 °C.

Table 1 shows that subtilisins were the best biocatalysts for the transesterification reaction. An important advantage of these enzymes is that scavengers, used with papain to prevent the oxidation of its active site, can be avoided. Furthermore, subtilisins are produced on an industrial scale for detergent formulations, so that they are readily available and inexpensive. Bromelain and trypsin, although specific for basic amino acids, turned out to be unable to catalyse this reaction. The high degree of acyl donor hydrolysis indicates that they have poor affinity for glycerol at the S1′ subsite.

Lipases were also inefficient biocatalysts for the synthesis of Boc-Arg-OGl·HCl **1**. Valivety *et al.*[2b] reported that some lipases, notably from *Candida antarctica*, readily accept N^{α}-(benzyloxycarbonyl)amino acids as substrates for esterification reactions with long aliphatic alcohols and diols. In the present case, as we will demonstrate below, the N^{α} protecting group of the arginine had a marked influence on the lipase reactivity for this particular amino acid.

Biocatalyst concentration and configuration

To assess the best process for the enzymatic synthesis of glyceryl esters, both possibilities, of free and deposited enzymes, were considered. On the basis of the aforedescribed results, subtilisins and papain were the biocatalysts of choice. The solubility of Protease N, Alcalase™ and papain in glycerol was ascertained, obtaining homogeneous solutions in all cases. As Table 2 shows, reactions using deposited enzyme resulted in higher Boc-Arg-OMe·HCl conversions and/or reaction rates than those using the enzyme in solution. These results are consistent with the fact that, in this reaction, deposition stabilizes the enzyme. Nevertheless, it was observed that the ratio of transesterification product : hydrolysed acyl donor, akin to the acyl donor partition into water and one of the primary OH groups of glycerol, was unfavourable using deposited enzymes. This was particularly significant with papain; using the enzyme deposited onto Celite™ the reaction was faster but the yield was lower than that with soluble enzyme. The same behaviour was observed with Alcalase™ (Table 2).

Table 1 Effect of biocatalyst on the transesterification reaction of Boc-Arg-OMe·HCl with glycerol[a]

Enzyme	Product yield[b] (%)	Acyl donor hydrolysis yield (%)
Alcalase	69	18
Protease N	65	10
Subtilisin BPN′	68	17
Trypsin	20	70
Bromelain	10	15
Lipase A	10	2
Lipase PS	12	2
Novozym 435	3	5

[a] Boc-Arg-OMe·HCl (500 mM) was dissolved in glycerol (1 ml) containing boric–borate 0.1 M, pH 8.2, buffer (10% v/v). Reactions were carried out at 50 °C. Enzymes deposited onto Celite™ (200 mg ml^{-1}, 100 mg enzyme g^{-1} Celite™) were used as catalysts. [b] After 48 h.

Thermodynamically controlled synthesis

The synthetic pathway leading to Boc-Arg-OGl·HCl **1** involved the preparation of the acyl donor Boc-Arg-OMe·HCl from either Boc-Arg-OH·HCl or H-Arg-OMe. For the sake of simplicity, we investigated if Boc-Arg-OH·HCl could be esterified directly under thermodynamically controlled conditions (Scheme 1B).

Initially, reactions were carried out at 50 °C in glycerol containing 10% v/v buffer (boric–borate, 0.1 M, pH 8.2 for papain and HCl-Tris, 50 mM, pH 7.8 for Protease N and Alcalase) using papain, Protease N and Alcalase deposited onto Celite™ as catalysts. Papain–Celite™ preparation gave 60% esterification yield in 48 h. Attempts to improve this yield by reducing the buffer concentration failed: at 5% v/v buffer, a 10% yield was achieved in 72 h. Mitin *et al.*[7] suggested that denaturation of papain may occur under these conditions. On the other hand, additions of buffer up to 15% v/v did not affect the yield substantially (58% after 48 h). Thus, the effect of buffer on the reaction performance was similar to that observed under kinetically controlled conditions. To our surprise, Protease N and Alcalase™ gave less than 2% of Boc-Arg-OGl·HCl **1**. Again, as we demonstrate below, the Boc protecting group exerted a strong influence on the reactivity of this substrate, in particular under thermodynamic control.

The esterification yield under thermodynamically controlled conditions depends crucially on the ionization equilibrium of the carboxylic group involved. Therefore, in the present case, the reaction pH should range between 3 and 4 according to the p*K* of the $^{\alpha}$COOH of the N^{α}-protected amino acid derivatives. Thus, enzymatic reactions were also conducted in glycerol containing citrate buffer, 0.1 M, pH 3.2 or 4.0 (10% v/v) using papain deposited onto Celite™ at the corresponding pH. Under these conditions, the yields dropped dramatically to 40% and 30%, respectively. The best result (60%) was achieved when the enzyme was deposited at pH 8.2. Braun and Kuhl[11] also found that pH 8.6 was the optimum for papain-catalysed esterification of N^{α}-protected amino acids in organic media. These results differ from the optimal pH (pH_{opt} 3.2) reported for papain-catalysed ester-bond formation in predominantly aqueous

Table 2 Effect of biocatalyst configuration on the transesterification reaction of Boc-Arg-OMe·HCl with glycerol[a]

Enzyme	Biocatalyst configuration (mg)	Reaction time (*t*/h)	Yield (%)	Acyl donor hydrolysis (%)	Substrate conversion (%)	Product : acyl donor hydrolysis ratio
Papain	Deposited (400)[b]	8	53	41	94	1.3
	Free (40)	48	70	23	93	3.0
Protease N	Deposited (400)[b]	48	70	17	87	4.1
	Free (40)	48	46	5	51	9.2
Alcalase	Deposited (400)[b]	48	70	23	93	3.0
	Free (40)[c]	48	59	6	66	9.8
	Free (66)[c]	48	70	8	78	8.8
	Free (80)[c]	48	76	6	82	12.7
	Free (132)[c]	48	79	14	92	9.8

[a] Boc-Arg-OMe·HCl (500 mM) was dissolved in glycerol (1 ml) containing the corresponding aqueous buffer solution (10% v/v; boric–borate 0.1 M, pH 8.2, for papain and Tris-HCl 50 mM, pH 7.8, for both Alcalase™ and Protease N). Reactions were carried out at 50 °C. [b] mg of enzyme-support preparation (100 mg enzyme g^{-1} Celite™, that is, 40 mg of enzyme in the reaction). [c] The enzyme was added as liquid preparation (66 mg protein ml^{-1} liquid preparation).

Table 3 Hydrolase-catalysed synthesis of N$^{\alpha}$-protected amino acid glyceryl ester derivatives[a]

N$^{\alpha}$-Protected amino acid glyceryl ester	Enzyme	Reaction yield (%)	Isolated yield (g) (% of theor.)
1	Papain	76	8 (55)
2	Papain	74	13 (67)
	Alcalase™	79	8 (59)
	Novozym 435™[b]	90	15 (80)
3	Papain	6	
	Alcalase™	19	
	Novozym 435™[b]	89	12 (73)
4	Papain	74	9 (53)
	Alcalase™	2	
	Novozym 435™[b]	6	
5	Papain[c]	61	10 (46)
	Alcalase™	10	
	Novozym 435™[b]	2	
6	Papain	72	13 (64)
	Alcalase™	8	
	Novozym 435™[b]	3	
7	Papain	12	
	Alcalase™	10	
	Novozym 435™[b]	3	
	Protease N[d]	87	10 (71)
	Papain[e]	62	

[a] Reaction conditions as described in Experimental section. The aqueous buffer content was 10% v/v unless otherwise stated. [b] The water content in the medium was 5% v/v. [c] The buffer content was 15% (v/v). [d] Reaction was carried out under kinetically controlled conditions (transesterification reaction) and anhydrous conditions. [e] Reaction was carried out under kinetically controlled conditions.

media.[7] There is evidence that in low-water systems the acid–base conditions may change due to, as in the present case, acidic substrates. Thus, the ionization state of the enzyme, akin to its activity, cannot be controlled exclusively by pH but also by the counter-ion.[12] It seems likely that papain deposition at pH 8.2 can ensure its optimal ionization state in low-water conditions and in the presence of acidic substrates.

Finally, it was observed that yields improved significantly when free papain (40 mg ml^{-1}) was used: 72% compared with 60% yield when using deposited enzyme. As in the kinetically controlled approach, the support seems to have a negative effect on the reaction yield.

Synthesis of *rac*-1-*O*-(*N*$^{\alpha}$-acetyl-L-aminoacyl)glycerol

The scope of the synthetic methodology developed for the preparation of **1** was extended to the other target amino acids. Crude and/or industrial protease preparations, namely papain, Alcalase™, Protease N and Novozym 435™, used as powder or liquid (straight from the bottle), were screened as putative biocatalysts. The syntheses were conducted first at analytical level and then on a preparative scale under the best reaction conditions. The results obtained are presented in Table 3. Important features are the following. Papain was a quite flexible catalyst for the synthesis of 1-*O*-(N$^{\alpha}$-protected aminoacyl)glycerol, being reactive for most of amino acids. The direct esterification under thermodynamically controlled conditions was used except for the preparation of **7**. In this case, Ac-Tyr-OH was found to be inactive for the enzymes assayed and kinetically controlled conditions had to be employed.

The negative influence of the support in papain-catalysed reactions with Boc-Arg-OX·HCl (X: H, Me) was confirmed using Ac-Arg-OH·HCl. Only a 22% yield of **2** was achieved using papain deposited onto Celite,™ compared with 74% using soluble enzyme. In contrast, the yields with glutamic acid, asparagine or glutamine were similar regardless of enzyme configuration.

In the previous section, we found that Alcalase and Novozym 435™ failed as catalyst for the synthesis of **1** using Boc-Arg-OX·HCl (X: H, Me) as acyl donor. Interestingly, Ac-Arg-OH turned out to be a good substrate for both Alcalase™ and Novozym 435™. As reported for chymotrypsin, the reactivity of these enzymes seemed largely affected by both geometry and hydrophobicity of the N$^{\alpha}$ protecting group of the amino acid.[13]

The acidic amino acids, Asp and Glu, showed a quite striking behaviour. Novozym 435™ gave the best yield in the synthesis of **3**, whereas it was found to be papain for **4**. A number of papers[14,15] reported the regioselective $^{\alpha}$COOH enzymatic ester synthesis and hydrolysis of the aspartic acid and glutamic acid derivatives. As expected, in the present case, both enzymes esterified selectively the $^{\alpha}$COOH groups of these amino acids. The esterification position was established by comparing the ^{13}C NMR spectra of the N$^{\alpha}$-acetylamino acids with the corresponding glyceryl ester derivatives. The α-ester substitution induced 1.18 and 1.46 ppm upfield shifts on the α-carbon of aspartic acid and glutamic acid, respectively (and no effect on the chemical shifts of β and γ carbons).[15]

In all the amino acid glyceryl ester synthesized, the presence of 2-*O*-regioisomer [2-*O*-(*N*$^{\alpha}$-acetyl-L-aminoacyl)-glycerol] (≈10%) was detected by ^{1}H NMR. The ^{1}H NMR signals were unequivocally assigned using the aspartic acid derivative as a model. In this case, both regioisomers, namely *rac*-1-*O*- (**4**) and 2-*O*-(*N*$^{\alpha}$-Ac-L-aspart-1-yl)glycerol, were isolated by HPLC ($t_{r1\text{-}O}$ = 7.5 min, $t_{r2\text{-}O}$ = 6.3 min; on a C-18 column, eluted isocratically with 0.1% v/v TFA in water). Formation of the ester bond in the secondary hydroxy group [2-*O*-(*N*$^{\alpha}$-Ac-L-aspart-1-yl)glycerol] resulted in a downfield shift of the proton at C2 of the glycerol moiety from δ 3.6 to δ 4.7 and a single signal at δ 3.4 corresponding to the four symmetric protons at C1 and C3. These NMR data helped us in identifying and quantifying the 2-*O*-regioisomers of the other amino acid glyceryl esters by NMR. The enzymatic esterification time course of aspartic acid by the glycerol, as in the esterification of the other amino acid derivatives, was followed by HPLC analysis. As mentioned above, in the case of aspartic acid HPLC baseline separation of both regioisomers was achieved. The chromatograms revealed that a major product was formed during the reaction time, which was identified as the *rac*-1-*O*-regioisomer. A minor peak appeared simultaneously, which was identified as the 2-*O*-regioisomer, constituting around 10% of the product at the end of the reaction (24 h). This percentage did not change during the reaction work-up and product purification. Hence, in agreement with the literature data,[16,17] this result indicated that the enzymes exhibited a preference towards

acylation of one of the primary hydroxy groups in the glycerol molecule (*i.e.*, alcohol groups in C1 or C3 positions). This suggested that the 2-regioisomer, although it could be produced enzymatically to some extent, it may be formed preferentially by simple 3(or 1) → 2 acyl migration similar to that observed in mono- and diglycerides[18] and monoesters of Boc-Ala-OH with propane-1,2-diol.[16]

It was ascertained that the enzymes used could not distinguish between the two enantiotopic hydroxymethyls of glycerol. The presence of both diastereoisomers was detected and quantified (approximately 50% of each) by ^{1}H NMR. The ^{1}H NMR spectra of diastereoisomeric amino acid glyceryl esters were distinguishable by the hydrogens of the C1 bearing the amino acid residue (ROCOCH_2CHOHCH$_2$OH).[19] At this stage, however, it was not possible to separate them or their derivatives (*i.e.* those obtained by acetylation or benzoylation of the free hydroxy groups) by chromatographic techniques. Hence, the specific assignation of the ^{1}H NMR signals to *S*,*R* or *S*,*S* diastereoisomers was not possible.

An important issue of the aforedescribed synthetic methodology was that of the purification procedures. All the products were highly soluble in water, so that simple solvent extractions were completely inefficient. Amino acid glyceryl derivatives such as **1**–**4**, containing charged amino acids, were purified by anion- or cation-exchange chromatography. Purification of uncharged compounds **5** and **6** was achieved using column chromatography on activated charcoal–Celite™ 50 : 50, whereas **7** was purified by means of hydrophobic interactions on either styrene–divinylbenzene or C-18-type stationary phases.

Experimental

Materials

Papain (EC 3.4.22.2) from papaya latex crude powder [1.7 U mg^{-1} of solid, one unit (U) will hydrolyse 1.0 μmol of N^{α}-benzoyl-L-arginine ethyl ester hydrochloride per minute (BAEE) at pH 6.2 at 25 °C], bromelain (EC 3.4.22.4) from pineapple stem (2100 U mg^{-1}, one unit will release 1.0 μmol of *p*-nitrophenol from N^{α}-Z-L-lysine-*p*-nitrophenyl ester per min at pH 4.6 at 25 °C), subtilisin BPN′ (EC 3.4.21.62) crystallized and lyophilysed (7.8 U mg^{-1} of solid) and trypsin (EC 3.4.21.4) from porcine pancreas (15,900 U mg^{-1} protein) crystallized, dialysed and lyophilysed were obtained from Sigma (St. Louis, MO, USA). *Bacillus subtilis* protease (Proteinase N) (EC 3.4.24.4) [7.3 U mg^{-1} of solid, one unit (U) corresponds to the amount of enzyme which liberates 1 μmol of folin-positive amino acid and peptides per minute at pH 7.0 and 37 °C using casein as substrate] was obtained from Fluka (Buchs, Switzerland). *Bacillus subtilis* protease (Alcalase™) (EC 3.4.21.62) (0.6 U mg^{-1}) and *Candida antarctica* lipase (Novozym 435™) (EC 3.1.1.3) (7000 PLU g-1, PLU: propyl laurate units) were a generous gift from Novo Nordisk A/S (Bagsvaerd, Denmark). *Aspergillus niger* lipase (lipase A™, 136,000 U g^{-1} Amano's method) and *Pseudomonas cepacia* lipase (lipase PS™, 32100 U g^{-1} Amano's method) were generously donated by Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). N^{α}-Acetyl-L-arginine (Ac-Arg-OH) [which was converted to N^{α}-acetyl-L-arginine hydrochloride (Ac-L-Arg-OH·HCl) by treatment with HCl], N^{α}-acetyl-L-glutamine (Ac-Gln-OH) and N^{α}-*tert*-butoxycarbonyl-L-arginine hydrochloride (Boc-Arg-OH·HCl) were obtained from Novabiochem (Läufelfingen, Switzerland). N^{α}-*tert*-Butoxycarbonyl-L-arginine methyl ester hydrochloride (Boc-Arg-OMe·HCl) was synthesized in our laboratory as described below. N^{α}-Acetyl-L-tyrosine ethyl ester was synthesized in our lab using the standard thionyl dichloride procedure. Glycerol, N^{α}-acetyl-L-glutamic acid (Ac-Glu-OH), N^{α}-acetyl-L-aspartic acid (Ac-Asp-OH), 1,4-dithio-DL-threitol (DTT) and Celite™ (type 545, particle size 26 μm; mean pore diameter 17 000 nm; specific surface area BET method 2.19 m^2 g^{-1}) were obtained from Fluka. Polyamide-6 (EP 700, particle size < 800 μm; mean pore diameter 50–300 nm; specific surface area BET method 8.4 m^2 g^{-1}) was a generous gift from Akzo (Obernburg, Germany). Solvents and other chemicals used in this work were of analytical grade.

Instrumentation

Mass spectrometric analysis were performed at the Servei d'Espectrometria de Masses of the University of Barcelona. Electrospray-mass spectrometric (ES-MS) analyses were recorded on a VG-Quattro system from Fisons Instruments (Altricham, UK). The carrier solution was water–CH_3CN (1 : 1) containing 1% (v/v) formic acid. Nuclear magnetic resonance (NMR) analyses were carried out at the Instituto de Investigaciones Químicas y Ambientales-CSIC. ^{1}H and ^{13}C NMR spectra were recorded with a Unity-300 spectrometer from Varian (Palo Alto, California, USA) for d_6-DMSO and CD_3OD solutions.

Methods

Enzyme adsorption. The general procedure for the deposition of the enzymes onto solid support materials was the following. An enzyme solution in the proper buffer (1 ml) was mixed thoroughly with the support (1 g). The mixture was then evaporated under vacuum overnight. For lipase A™, lipase PS™, Alcalase™, proteinase N, subtilisin BPN′ and trypsin 100 mg of enzyme g^{-1} of Celite™ were prepared using 50 mM HCl-Tris, pH 7.8, buffer. For bromelain and papain, the enzyme (100 mg) and DTT (50 mg) were dissolved in 0.1 M boric–borate pH 8.2 buffer and mixed with Polyamide or Celite™ depending on the experiment (1 g).

HPLC. The amount of reactants and products in the enzymatic reactions was measured by HPLC analysis. HPLC analyses were performed on a Merck-Hitachi (Darmstadt, Germany) Lichrograph system using a Lichrocart 250–4 HPLC cartridge, 250 × 4 mm filled with Lichrosphere® 100, RP-18, 5 μm (Merck). Samples (8 μl) were withdrawn from the reaction medium, mixed with acetic acid (8 μl) to stop any further enzymatic reaction, evaporated under vacuum, and dissolved in HPLC eluent before analysis. Quantitative analysis was performed from peak areas by means of the external standard method. Preparative HPLC runs were performed on a Waters (Milford, MA, USA) Prep LC 4000 pumping system and a Waters PrePack® 1000 module fitted with a PrePack® (Waters) column (47 × 300 mm) filled with Bondapack C18, 300 Å, 15–20 μm stationary phase for the purification of Ac-Tyr-OGl.

Synthesis of N^{α}-*tert*-butoxycarbonyl-L-arginine methyl ester hydrochloride (Boc-Arg-OMe·HCl). Arginine methyl ester dihydrochloride (22.0 g, 84.23 mmol) was dispersed in dimethylformamide (DMF) (250 ml). Then, triethylamine (14 ml, 101.1 mmol) was added dropwise and the reaction mixture was stirred overnight at 25 °C. The reaction mixture was filtered and the solid collected was identified as triethylamine hydrochloride. Then, the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in CH_3CN (200 ml), and di-*tert*-butyl dicarbonate (23.9 g, 109.5 mmol) was added dropwise over a period of 15 min. The reaction was allowed to proceed at 25 °C for 24 h. Then, the solvent was removed under vacuum. The solid obtained was dissolved in water and washed successively with ethyl acetate (3 × 150 ml) and diethyl ether (3 × 150 ml). The aqueous layer, containing the product, was freeze dried, yielding a highly hygroscopic white solid (20.5 g, 74%), δ_H (300 MHz; d_6-DMSO; 45 °C) 7.87 (1H, s, CON*H*CH), 7.24 [5H, m, NHC(NH_2)$_2$], 3.93 (1H, m, NHC*H*COOCH$_3$), 3.61 (3H, s, COCH_3), 3.04 (4H, m,

$CH_2CH_2CH_2NH$), 1.51 (2H, m, $CHCH_2CH_2CH_2NH$), 1.39 (9H, s, $(CH_3)_3CO$); δ_C (75 MHz; d_6-DMSO; 45 °C): 172.69 ($COOCH_3$), 157.06 (C=N guanidine group), 155.37 (O-C-O-NH), 78.14 [$(CH_3)_3C$-O], 53.05 ($NHCHC{=}O$), 51.54 (OCH_3), 45.27–25.07 (CH_2), 28.02 [$(CH_3)_3C$-O]; *m*/*z* 289.1 $([M + H])^+$. $C_{12}H_{25}N_4O_4$ requires *m*/*z*, 289.3).

Enzymatic reactions. Reactions on an analytical scale were carried out in 5 ml closed flask vessels placed on a reciprocal shaker (200 rpm) at 50 °C. The amino acid derivative (0.5 mmol) was dissolved in glycerol (13.7 mmol in 1 ml), containing the corresponding buffer (0–15% v/v depending of the experiment), at 50 °C. The reactions were started by the addition of the enzyme, deposited onto a solid support or straight from the bottle depending on the experiment. When the reactions were catalysed by soluble papain and bromelain, the reaction medium contained DTT (50% of the amount of enzyme). Moreover, reactions with these two enzymes were performed under argon atmosphere.

HPLC reaction monitoring. Synthesis of Boc-Arg-OGl·HCl. Solvent system: solvent [A]: aq. triethylamine–phosphoric acid buffer, pH 6.0 (TEAP pH 6.0) in water; solvent [B]: TEAP, pH 6.0–CH_3CN 1 : 4; gradient elution from 5 to 60% B in 30 min; retention factor (k') for each product: Boc-Arg-OH (4.9), Boc-Arg-OMe (8.5), Boc-Arg-OGl (6.1).

Synthesis of N^{α}-acetylamino acid derivatives. Solvent system: solvent [A]: 0.1% (v/v) trifluoroacetic acid (TFA) in water, solvent [B]: 0.085% (v/v) TFA in water–CH_3CN 1 : 4; isocratic elution 0% B; k'-values for each product: Ac-Arg-OH (2.6), Ac-Arg-OGl (5.2), Ac-Asp-OH (1.2), Ac-Asp-OGl (2.6), Ac-Glu-OH (2.4), Ac-Glu-OGl (5.2), Ac-Asn-OH (0.7), Ac-Asn-OGl (1.5), Ac-Gln-OH (0.9), Ac-Gln-OGl (2.2).

Synthesis of N^{α}-Ac-Tyr-OGl. Solvent system: solvent [A]: 0.05% v/v CH_3COOH in water, solvent [B]: 0.05% v/v CH_3-$COOH$ in water–CH_3CN 1 : 4; gradient elution from 10 to 70% B in 30 min; k'-values for each product: Ac-Tyr-OH (3.2), Ac-Tyr-OEt (7.7), Ac-Tyr-OGl (4.0). In all cases, the flow rate was 1 ml min^{-1}, with UV detection at 215 nm.

Enzymatic reactions at preparative level. Scale-up of the reactions was made on the basis of approximately 10 g of pure product, considering the reaction and purification yields obtained at analytical level. The N^{α}-protected amino acid (35–70 mmol) was dissolved in glycerol (70–175 ml) at 50 °C, containing the corresponding amount of buffer (0–15% v/v) and the scavenger (DTT in the case of papain, 1.5–3.5 g). The reactions were initiated by the addition of the enzyme (see Table 3) and monitored by HPLC. At the end, the reaction mixture was diluted three times with a mixture of MeOH–acetic acid 4 : 1 to stop any further enzymatic reaction. Upon the addition of MeOH–acetic acid, most of the enzyme became denatured and a fluffy solid appeared in the solution. This was especially visible in the reactions carried out using the enzyme in solution. It was found that filtration through Celite™ was an effective way to remove the precipitate and eliminate all possible enzymatic activity. The filtrate obtained was evaporated under vacuum to eliminate the methanol and acetic acid. Then, the oily residue was dissolved with plain, deionized water and purified as described above. In the case of arginine derivatives, the final water solution was adjusted to pH 4 with 0.2 M NaOH before purification.

Purification procedures. *Ion-exchange chromatography.* General: Bulk stationary phases MacroPrep High S Support, 50 μm strong cation-exchange resin (Bio-Rad, Hercules, CA, USA) and Analytical Grade anion-exchange resin AG 3-X4, 100–200 mesh (Bio-Rad), were packed into glass columns of the flash-chromatography type, to a final bed volume of 370 ml, bed dimensions 190 × 50 mm. The eluent was passed through the stationary phase by nitrogen pressure, at a flow rate of 60–80 ml min^{-1}, 3–4 cm min^{-1}. Elution conditions for each product using this technique are the following.

Boc-Arg-OGl·HCl and Ac-Arg-OGl·HCl. The resin (MacroPrep High S Support) was equilibrated initially with 50 mM phosphate buffer, pH 5.7 (1.5 l, 4 bed volumes), and washed subsequently with plain, deionized water (2.4 l, 6.5 bed volumes). Then, aliquots of the residue containing 6 or 7 g of crude Boc-Arg-OGl·HCl or Ac-Arg-OGl·HCl, respectively were loaded. First, the glycerol and Boc-Arg-OH·HCl or Ac-Arg-OH·HCl were eliminated by washing The resin with 4.4 l (12 bed volumes) of plain, deionized water; at this pH both arginine derivatives have the COOH group unprotonated and consequently are electrically neutral. Then, the target products were eluted isocratically by 0.74 l (2 bed volumes) of 250 mM NaCl in water–EtOH 3 : 2. Products were desalted by precipitation with anhydrous ethanol.

Ac-Asp-OGl and Ac-Glu-OGl.. First, the resin (anion-exchange resin AG 3-X4) was equilibrated with 1.0 M acetate buffer, pH 7.2, and washed subsequently with plain, deionized water. Then, aliquots of the residue containing 11 g of crude Ac-Asp-OGl or 6 g of crude Ac-Glu-OGl were loaded. After that, the glycerol was eliminated by washing the resin with 5.6 l (15 bed volumes) of plain, deionized water. Elution of the products were achieved isocratically by 1.5 l (4 bed volumes) of aq. 0.5 M acetic acid.

Celite™/activated charcoal chromatography. This chromatographic support is used extensively for the separation of polyhydroxylated compounds such as oligosaccharides from monosaccharides.[20] A mixture of Celite™ and activated charcoal 1 : 1 (150–160 g of each) was packed in a Buchner fritted funnel (10 × 13 cm, with a fine-porosity fritted-glass disk) to a final bed volume of 940–1000 ml. The eluent was passed through the stationary phase by vacuum. The chromatographic system was treated initially with an aqueous solution of acetic acid (10% v/v; 1.5 l) and washed subsequently with plain, deionized water to pH 5. Then, aliquots of the residue containing 3.75 g of crude Ac-Asn-OGl or 7 g of crude Ac-Gln-OGl were loaded. The glycerol was eliminated by washing the support with plain, deionized water (9 l, 10 bed volumes). Elution of Ac-Asn-OGl or Ac-Gln-OGl was achieved isocratically by 1.25 l (1.2 bed volumes) and 2.7 l (2.7 bed volumes) of MeOH–water 13 : 7, respectively.

Reversed-phase chromatography. In this case either C-18 or bulk porous polymeric media based on styrenic polymers (Amberchrom CG-161M, Montgomeryville, PA, USA) were used as stationary phases. Both were effective for the purification of the most hydrophobic, Ac-Tyr-OGl, derivative. For the C-18 reversed phase the procedure was the following. Aliquots of the residue containing crude Ac-Tyr-OGl (1.5 g) were loaded onto the preparative PrePack® (Waters) column (47 × 300 mm) filled with Bondapack C_{18}, 300 Å; 15–20 μm cartridge, and eluted using an aq. acetic acid solution (0.05% v/v) and gradient elution from 0 to 16% CH_3CN in 20 min. The flow rate was 100 ml min^{-1} and the products were detected at either 215 or 225 nm. Using the porous styrenic polymer the conditions were the following. Aliquots of the residue containing crude Ac-Tyr-OGl (9 g) was loaded onto the Amberchrom CG-161M resin (370 ml, bed dimensions 190 × 50 mm packed on flash-chromatography-type column), and eluted using water and gradient elution from 0 to 80% EtOH in 60 min.

In all cases, the operation was repeated until the whole crude was consumed. Analysis of the fractions was accomplished under analytical RP-chromatography using the same elution conditions, flow rate, and detection as previously described. Pure fractions were pooled and lyophilised. Purity of the amino acid glyceryl ester obtained was assessed by HPLC. NMR and mass spectroscopy data for each compound are given below.

***rac*-1-*O*-(N^{α}-Boc-L-arginyl)glycerol hydrochloride (Boc-Arg-OGl·HCl) 1.** Purity 92.3% by HPLC; δ_H (300 MHz; d_6-DMSO;

45 °C) 8.18 (1 H, d, CONHCH), 7.33 [5 H, m, $NHC(NH_2)_2$], 4.12–3.87 (3 H, m, $NHCHCOOCH_2$), 3.64 (1 H, quintet, *J* 5.4, $COOCH_2CHOHCH_2OH$), 3.34 (2 H, d, *J* 6, $CHOHCH_2OH$), 3.06 (2 H, t, CH_2NH), 1.54 [4 H, m, $CH(CH_2)_2$], 1.3 [9H, s, $(CH_3)_3CO$]; δ_C (75 MHz; d_6-DMSO; 45 °C) 172.15 (CHCOO), 156.96 (C=N guanidine function), 155.41 (OCONH), 78.15 [$(CH_3)_3CO$), 69.09 (CHOH), 65.87 (OCH_2CHOH), 62.41 (CH_2OH), 53.15 (NHCHCO), 40.33 (CH_2NH), 28.04 [$(CH_3)_3$-CO], 27.85 (CH_2), 24.91 (CH_2); *m*/*z* (ES) 348.8 ($[M + H]^+$. $C_{14}H_{29}N_4O_6$ requires *m*/*z*, 349.2).

***rac*-1-*O*-(*N*$^{\alpha}$-Ac-L-arginyl)glycerol hydrochloride (Ac-Arg-OGl·HCl) 2.** Purity 97.9% by HPLC; δ_H (300 MHz; d_6-DMSO; 45 °C) 8.26 (1 H, d, CONHCH), 7.85 [1 H, t, $CH_2NHC(CH_2)_2$], 7.20 [4 H, s, $CH_2NHC(CH_2)_2$], 4.23 (4 H, m, $NHCH_2CH_2$), 4.09 (0.5 H,dd, *J* 11.1 and 4.5, $COOCH_2CHOH$, diaster. I), 4.03 (0.5 H, dd, *J* 11.1 and 4.8, $COOCH_2CHOH$, diaster. II), 3.93 (0.5 H, dd, *J* 11.1 and 6.3, $COOCH_2CHOH$, diaster. II), 3.91 (0.5 H, dd, *J* 11.1 and 6.3, $COOCH_2CHOH$, diaster. I), 3.64 (1 H, quintet, *J* 5.7, $COOCH_2CHOHCH_2OH$), 3.35 (2 H, br s, *J* 6.3, $CHOHCH_2OH$), 3.11 (1 H, m, NHCHCO), 1.85 (3 H, s, CH_3CO); 1.73–1.48 (2 H, m, CH_2CH); δ_C (75 MHz; d_6-DMSO; 45 °C) 171.81 (CHCOO), 169.44 (CH_3CO), 157.06 (C=N guanidine function), 69.15 (CHOH), 65.92 (OCH_2CHOH), 62.48 (CH_2OH), 51.61 (NHCHCO), 40.33 (CH_2NH), 27.95 (CH_2), 24.94 (CH_2), 22.12 (CH_3); *m*/*z* (ES) 292.1 ($[M + H]^+$. $SC_{11}H_{23}N_4O_5$ requires *m*/*z*, 291.2).

***rac*-1-(*O*-(*N*$^{\alpha}$-Ac-L-aspart-1-yl)glycerol (Ac-Asp-OGl) 3.** Purity 95.5% by HPLC; δ_H (300 MHz; d_6-DMSO; 45 °C) 12.30 (1 H, s, CH_2COOH), 8.11 (1 H, d, CONHCH), 4.56 (1 H, m, NHCHCO), 4.06 (0.5 H, dd, *J* 11.1 and 4.2, $COOCH_2CHOH$, diaster. I), 4.05 (0.5 H, dd, *J* 11.1 and 4.5, $COOCH_2CHOH$, diaster. II), 3.92 (1 H, dd, *J* 11.4 and 4.2, $COOCH_2CHOH$, diaster. I + II), 3.63 (1 H, quintet, *J* 6, $CH_2CHOHCH_2$), 3.46 (2 H, br s, *J* 7.8, $CHOHCH_2OH$), 2.80–2.61 (2 H, m, CH_2-COOH), 1.82 (3 H, s, CH_3CO); δ_H (300 MHz; CD_3OD; 20 °C) 5.19 (1H, m, NHCHCO), 4.61 (0.5 H, dd, *J* 11.4 and 4.2, $COOCH_2CHOH$, diaster. I), 4.60 (0.5 H, dd, *J* 11.1 and 4.2, $COOCH_2CHOH$, diaster. II), 4.52 (0.5 H, dd, *J* 11.4 and 6.0, $COOCH_2CHOH$, diaster. II), 4.51 (0.5 H, dd, *J* 11.4 and 4.2, $COOCH_2CHOH$, diaster I), 4.25 (1H, quintet, *J* 6, $COOCH_2$-$CHOHCH_2OH$), 3.9 (2 H, br s, *J* 7.8, $CHOHCH_2OH$), 3.36–3.28 (2H, m, CH_2COOH), 2.40 (3 H, s, CH_3CO); δ_C (75 MHz; d_6-DMSO; 45 °C) 172.38 (β-COOH), 170.28 (CHCOO), 169.96 (CH_3CO), 69.24 (CHOH), 66.09 (OCH_2CHOH), 62.61 (CH_2OH), 48.54 (NHCHCO), 36.03 (CH_2COOH), 22.42 (CH_3); *m*/*z* (ES) 249.9 ($[M + H]^+$. $C_9H_{16}N_1O_7$ requires *m*/*z*, 250.2).

***rac*-1-*O*-(*N*$^{\alpha}$-Ac-L-glutam-1-yl)glycerol (Ac-Glu-OGl) 4.** Purity 98.8% by HPLC; δ_H (300 MHz; d_6-DMSO; 45 °C) 12.21 (1 H, s, COOH), 8.21 (1 H, d, CONHCH), 4.27 (1 H, m, NHCHCO), 4.09 (0.5 H, dd, *J* 11.1 and 4.5, $COOCH_2CHOH$, diaster. I), 4.04 (0.5 H, dd, *J* 11.4 and 4.5, $COOCH_2CHOH$, diaster. II), 3.97 (0.5 H, dd, *J* 10.8 and 6.3, $COOCH_2CHOH$, diaster. II), 3.92 (0.5 H, dd, *J* 11.1 and 6.3, $COOCH_2CHOH$, diaster. I), 3.63 (1 H, quintet, *J* 5.7, $COOCH_2CHOHCH_2OH$), 3.35 (2 H, br s, *J* 5.1, $CHOHCH_2OH$), 2.26 (2 H, m, CH_2-COOH), 1.94 (2 H, m, CH_2COOH), 1.83 (3 H, s, CH_3); δ_C (75 MHz; d_6-DMSO; 45 °C) 173.44 (γ-COOH), 171.71 (CHCOO), 169.36 (CH_3CO), 69.15 (CHOH), 65.90 (OCH_2CHOH), 62.49 (CH_2OH), 51.24 (NHCHCO), 29.86 (CH_2 COOH), 26.25 (CH_2), 22.10 (CH_3); *m*/*z* (ES) 263.7 ($[M + H]^+$. $C_{10}H_{18}N_1O_7$ requires *m*/*z*, 264.1).

***rac*-1-*O*-(*N*$^{\alpha}$-Ac-L-asparaginyl)glycerol (Ac-Asn-OGl) 5.** Purity 93.3% by HPLC; δ_H (300 MHz; d_6-DMSO; 45 °C): 8.06 (1 H, d, CONHCH), 7.29 (1 H, s, $CONH_2$), 6.81 (1 H, s, $CONH_2$), 4.57 (1 H, m, NHCHCO), 4.06 (0.5 H, dd, *J* 10.8 and 4.5, $COOCH_2CHOH$, diaster. I), 4.03 (0.5 H, dd, *J* 10.8 and 4.8, $COOCH_2CHOH$, diaster. II), 3.95 (0.5 H, dd, *J* 11.1 and 6.0, $COOCH_2CHOH$, diaster. II), 3.92 (0.5 H, dd, *J* 11.1 and 6.0, $COOCH_2CHOH$, diaster. I), 3.63 (1H, quintet, *J* 5.7, $COOCH_2CHOHCH_2OH$), 3.35 (2 H, br s, *J* 5.4, $CHOHCH_2$-OH), 2.47 (2 H, m, H_2OCCH_2), 1.82 (3 H, s, CH_3); δ_C (75 MHz; d_6-DMSO; 45 °C) 171.30 ($CONH_2$), 170.88 (CHCOO), 169.11 (CH_3CO), 69.15 (CHOH), 65.98 (OCH_2CHOH), 62.48 (CH_2OH), 48.89 (NHCHCO), 36.69 (CH_2COOH), 22.18 (CH_3); *m*/*z* (ES) 249.7 ($[M + H]^+$. $C_9H_{17}N_2O_6$ requires *m*/*z*, 249.1).

***rac*-1-*O*-(*N*$^{\alpha}$-Ac-L-glutaminyl)glycerol (Ac-Gln-OGl) 6.** Purity 90.9% by HPLC; δ_H (300 MHz, d_6-DMSO; 45 °C) 8.14 (1H, d, CONHCH), 7.17 (1 H, s, $CONH_2$), 6.67 (1 H, s, $CONH_2$), 4.22 (1 H, m, NHCHCO), 4.09 (0.5 H, dd, *J* 10.8 and 4.2, $COOCH_2CHOH$, diaster. I), 4.03 (0.5 H, dd, *J* 11.1 and 4.5, $COOCH_2CHOH$, diaster. II), 3.97 (0.5 H, dd, *J* 11.1 and 6.3, $COOCH_2CHOH$, diaster. II), 3.92 (0.5 H, dd, *J* 11.1 and 6.0, $COOCH_2CHOH$, diaster. I), 3.64 (1 H, quintet, *J* 5.7, $COOCH_2CHOHCH_2OH$), 3.36 (2 H, br s, *J* 5.4, $CHOHCH_2OH$), 2.12 (2 H, m, H_2NCOCH_2), 1.97 (2 H, m, CH_2CH), 1.84 (3 H, s, CH_3); δ_C (75 MHz; d_6-DMSO; 45 °C) 173.23 (γ-$CONH_2$), 171.86 (CHCOO), 169.32 (CH_3CO), 69.17 (CHOH), 65.86 (OCH_2CHOH), 62.50 (CH_2OH), 51.62 (NHCHCO), 31.10 (CH_2CONH_2), 26.74 (CH_2), 22.13 (CH_3); *m*/*z* (ES) 263.0 ($[M + H]^+$. $C_{10}H_{19}N_2O_6$ requires *m*/*z*, 263.1).

***rac*-1-*O*-(*N*$^{\alpha}$-Ac-L-tyrosinyl)glycerol (Ac-Tyr-OGl) 7.** Purity 99.2% by HPLC; δ_H (300 MHz, d_6-DMSO; 45 °C) 9.09 [1 H, s, C=C(OH)C], 8.10 (1 H, d, CONHCH), 6.98 [2 H, d, HC=C(CH_2)C=CH], 6.66 [2 H, d, HC=C(OH)C=CH], 4.49 (1 H, m, NHCHCO), 4.04 (0.5 H, dd, *J* 11.1 and 4.5, $COOCH_2CHOH$, diaster. I), 4.03 (0.5 H, dd, *J* 10.8 and 4.2, $COOCH_2CHOH$, diaster. II), 3.93 (0.5 H, dd, *J* 10.8 and 6.0, $COOCH_2CHOH$, diaster. II), 3.93 (0.5 H, dd, *J* 10.8 and 6.0, $COOCH_2CHOH$, diaster. I), 3.62 (1 H, quintet, *J* 5.1, $COOCH_2CHOHCH_2OH$), 3.33 (2 H, br s, *J* 5.1, $CHOHCH_2$-OH), 2.95–2.71 (2 H, m, $CHCH_2$), 1.79 (3 H, s, CH_3); δ_C (75 MHz, d_6-DMSO; 45 °C) 171.57 (CHCOO), 169.19 (CH_3CO), 155.79 (C=COH), 129.79 (CH_2C=C), 127.22 (CH_2C=C), 114.94 [C=C(OH)C], 69.13 (CHOH), 65.90 (OCH_2CHOH), 62.53 (CH_2OH), 53.78 (NHCH CO), 35.98 (CH_2), 22.13 (CH_3); *m*/*z*, (ES) 298.2 ($[M + H]^+$. $C_{14}H_{20}NO_6$ requires *m*/*z* 298.1).

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