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A New Simple Route to the Synthesis of Protease Substrates in Ice

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Abstract: The serine proteases α–chymotrypsin and trypsin and the cysteine protease papain were used to catalyze the synthesis of N^{α}-protected di- and tripeptide ester substrates by acyl transfer of the corresponding N^{α}-protected amino acid esters to various amino acid- or dipeptide esters. The very simple and economical strategy benefits from performing the enzymatic reaction in frozen-aqueous systems. The products, which can further act as specific substrates for several important proteases, are obtainable in high yields and can be isolated easily. The results show an extraordinary shift in S' specificty of the proteases in frozen aqueous systems and are of general importance for protease-directed step-by-step peptide synthesis with a minimal protection/deprotection techniques.

1. INTRODUCTION

Although proteases have been used as practical biocatalysts in peptide synthesis[1], the substrate specificity is so far, however, a serious limitation which prevents the enzymatic approach from being universally applicable. Therefore studying enzyme/substrate interactions by hydrolysis or acyl transfer studies is of great importance for broadening the general use of proteases[2]. Such studies require the application of specific, fine-tuned substrates which should correspond to the subsite specificity. They have to be soluble enough to provide a high turnover and should be easy to obtain. However, chemical synthesis of acyl donor ester substrates is a tedious procedure, especially if trifunctional amino acids are involved. In particular, this is valid for arginine- and lysine-containing peptides which require extensive and, in the case of arginine, expensive side-chain protection.

In this paper we present a very simple and economical strategy to synthesize N^{α}-protected di- and tripeptide ester substrates by acyl transfer of the corresponding N^{α}-prot ected amino acid esters to various amino acid- or dipeptide esters with the proteases α–chymotrypsin, trypsin and papain. These products can further act as specific substrates for several important proteases. Normally, the nucleophile efficiency of amino acid esters is significantly decreased compared with corresponding amino acid amides[3]. Our procedure takes

advantage from the fact that freezing aqueous reaction mixtures can drastically increase the yield in protease-catalyzed peptide synthesis[4]. The benefits of this strategy are high yields, suppressed secondary hydrolysis effects, an easy work-up procedure and mild reaction conditions. In order to demonstrate the practical scope of the method all protease substrates were synthesized in preparative scale.

RESULTS AND DISCUSSION

1. Selection of the substrates. Our main interest in synthesizing new protease substrates is directed to the S_1 preference (subsite nomenclature according to Schechter and Berger[5]) for basic amino acid residues of the serine protease trypsin[6] and the cysteine protease clostripain[7]. Therefore, acyl transfer to arginine- or lysine alkylesters using α–chymotrypsin with regard to its strong preference for basic residues in the P'_1 position should be the method of choice (Table 1). As N–terminal protecting groups acetyl-, solubilizing α-betainyl-($(CH_3)_3N^+$-CH_2-CO-) and benzoyl- were chosen. Furthermore, carboxamidomethyl-(Cam-)esters, which are well known as non-specific trypsin substrates[8], have been utilized as nucleophiles (H-Phe-OCam and H-Ala-Phe-OCam) in papain- and trypsin-catalyzed syntheses and compared with H-Phe-OMe. Trypsin and papain are suitable for the formation of Arg-Xaa-bonds, however papain prefers hydrophobic residues in P_2 position. Therefore, Bz-Arg-OEt could be interpreted as a well accepted substrate for papain. Looking at the product Bz-Arg-Ala-Phe-OCam initial experiments with both enzymes resulted in a significant higher yield using trypsin as the biocatalyst.

Table 1. Yields of Synthesized Protease Substrates by Different Proteases in Ice

Acyl donor	Nucleophile	Protease	Product	Yield[a] [%]
Bet-Phe-OEt	H-Arg-OEt	α-Chymotrypsin	Bet-Phe-Arg-OEt	63
Bet-Phe-OEt	H-Arg-OPr	α-Chymotrypsin	Bet-Phe-Arg-OPr	82
Ac-Tyr-OEt	H-Arg-OEt	α-Chymotrypsin	Ac-Tyr-Arg-OEt	81
Ac-Tyr-OEt	H-Arg-OPr	α-Chymotrypsin	Ac-Tyr-Arg-OPr	88
Bet-Phe-OEt	H-Lys-OEt	α-Chymotrypsin	Bet-Phe-Lys-OEt	42
Ac-Tyr-OEt	H-Lys-OEt	α-Chymotrypsin	Ac-Tyr-Lys-OEt	75
Bz-Arg-OEt	H-Phe-OMe	Papain	Bz-Arg-Phe-OMe	57
Bz-Arg-OEt	H-Phe-OCam	Papain	Bz-Arg-Phe-OCam	66
Bz-Arg-OEt	H-Ala-Phe-OCam	Trypsin	Bz-Arg-Ala-Phe-OCam	27

[a] The yields correspond to preparative syntheses in 0.2-1.0 mMol range.

2. Optimization of the reaction conditions. Under conventional reaction conditions serine and cysteine proteases require amino acid amides or peptides as nucleophiles in acyl transfer reactions because of acting as an endopeptidase. However, recently it was shown that α-chymotrypsin is able to act as reverse carboxypeptidase catalyzing coupling of free amino acids as nucleophilic components[9]. The use of esters as nucleophiles is, as mentioned above, possible but with a drastically decreased efficiency[3]. As model systems for our studies we used the acyl transfer of Bet-Phe-OEt to H-Arg-OR (R corresponds to methyl, ethyl and propyl, respectively). The results in frozen aqueous systems were compared with those in aqueous system at room temperature and in organic solvents (Table 2).

Table 2. Yields of the Acyl Transfer of Bet-Phe-OEt to Various Arginine Esters in Different Media

Nucleophile[a]	Aqueous System[b] Yield [%]		Organic Solvent[c] Yield [%]	Frozen Aqueous System[d]
	pH 9	pH 10	(Acetonitrile)	Yield [%]
H-Arg-OMe	0	0	0	57
H-Arg-OEt	9	10	0	63
H-Arg-OPr	n.d.	n.d.	n.d.	82

Reaction conditions: [a]usually as hydrochlorides, neutralization by addition of NaOH equivalents. [b] ϑ=25°C; [Bet-Phe-OEt]=10mM; [H-Arg-OR]=100mM; [CT]=0.12μM; 0.2M Na_2CO_3/$NaHCO_3$ buffer. [c] ϑ=25°C; [Bet-Phe-OEt]=100mM; [H-Arg-OR]=200mM; [CT]=50μM; 0.2M Na_2CO_3/$NaHCO_3$ buffer pH 10; [ACN]=90/95/98%(v/v); H-Arg-OMexHCl was neutalized by the addition of 1 equivalent triethylamine. [d] ϑ=-25°C; [Bet-Phe-OEt]=40mM; [H-Arg-OR]=100mM; [CT]=0.6μM; 0.2M Na_2CO_3 / $NaHCO_3$ buffer pH 10, [NaCl]=0.2M. n.d. not detected.

It is clearly demonstrated that, in contrast to the reactions in aqueous system and in organic solvents, high yields could be obtained in frozen aqueous systems. The application of organic solvents with low-water content in chymotrypsin-catalyzed peptide synthesis as described for several dipeptide syntheses[10] was not successful. Decreasing the water concentration by using micro-aqueous monophasic organic solvents was hoped to result in prevention of substrate ester and nucleophile hydrolysis as well as a change in enzyme specificity. The latter is obviously observed in frozen aqueous systems. The reaction temperatures of -15°C and -25°C, respectively, were proved to be optimally and leads usually to the highest yield. From Figure 1 it can be concluded that in frozen aqueous systems the enzymatic reaction proceeds with low rate constant. For practical purposes this behaviour is favourable because it results in drastical suppression of unwanted enzymatic hydrolysis in between enzyme addition and shock freezing.

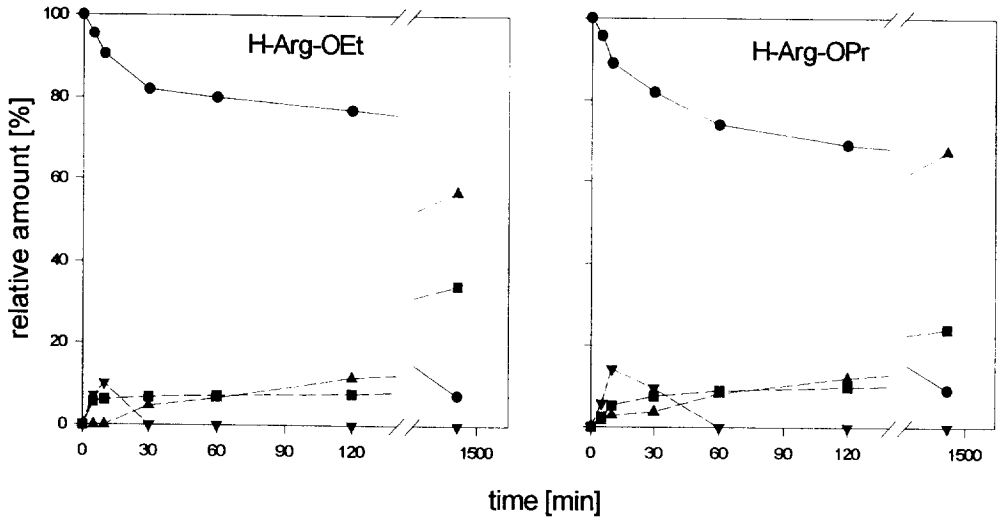

Figure 1. Time course of the α-chymotrypsin-catalyzed reaction of Bet-Phe-OEt with H-Arg-OEt and H-Arg-OPr (pH 10; ϑ= -25°C; I=0.2 M; [Bet-Phe-OEt]=10 mM; [HN]=100 mM; [CT]=0.36 μM)
(-•-) Bet-Phe-OEt; (-▲-) Bet-Phe-Arg-OEt/Bet-Phe-Arg-OPr; (-■-) Bet-Phe-OH; (-▼-) by-products

In order to synthesize mg-amounts of the substrates it was necessary to scale up from the analytical approach. This was achieved by five times increasing the total concentration of the educts which was proved to be without influence on the aminolysis/hydrolysis ratio of the synthetic course at the same reaction temperature.

Generally, at temperatures down to -40°C a dramatically improved ratio between aminolysis and hydrolysis of the acyl enzyme could be observed. As a reason for this yield-increasing effect the „freeze-concentration model" has been postulated[4b]. This behavior can be explained by the assumption that freezing the reaction mixture results in an effective increase in concentration of the reactants in the remaining unfrozen liquid phase, which is in equilibrium with the ice crystals. NMR-relaxion time measurements showed that in this liquid phase the nucleophiles are concentrated about fifty times higher than in the aqueous system (Ullmann.G and Jakubke H.-D., unpublished results).

The most remarkable result in using esters as nucleophiles in ice is based on a dramatically changed S' specificity of the enzyme. For the first time this surprising fact was established in α-chymotrypsin-catalyzed acyl transfer from Mal-Phe-OMe to H-Leu-OMe and H-Gly-OMe in analytical scale[11]. From structure-function relationship studies in the S' subsites of serine proteases based on superimposed structures of complexes between enzymes and inhibitors it was concluded that one of the most important contact in the S' subsites is a hydrogen bond between O(Phe-41) and HN(P'_2)[12]. This explains the normally significantly decreased acyl

transfer efficiency of amino acid esters which are unable to act as a hydrogen bond donor in P'_2. In frozen aqueous systems the importance of hydrogen bonds is postulated to be overlayed by anisotopic forces. Another result which supports the hypothesis that the „freeze-concentration model" cannot be responsible alone for these effects is a relatively drastical change of the S' subsite specificity of trypsin in frozen aqueous medium. It was possible to get high yields with Glu-NH_2 as nucleophile, which is almost not accepted by trypsin under normal reaction conditions[13]. Frozen state enzymology opens completely new possibilities in enzymatic peptide synthesis. The simplest strategy of peptide bond formation which cannot be performed by chemical methods is using N-terminal free amino acid or peptide esters as acyl donors[14].

The results presented in this paper reinforce this extraordinary shift in S' specificty and extend its appearance towards cysteine proteases. Consequently, the data indicate a general importance both for enzymatic peptide synthesis and also for biophysical considerations. The use of an amino acid ester as nucleophile in a protease-catalyzed peptide synthesis reaction is very attractive, since the resulting peptide ester can serve as acyl donor for the subsequent protease-directed reaction step. Thus the method of freezing opens the way to a protease-directed step-by-step peptide synthesis with a minimal protection/deprotection techniques.

EXPERIMENTAL

Chemicals. α-chymotrypsin (EC 3.4.21.1.) and papain (EC 3.4.22.2) were products of Merck (bovine pancreas; Darmstadt, Germany) and β-trypsin (EC 3.4.21.4.) of Serva (bovine pancreas) and were used without further purification. The acyl donor esters Bz-Arg-OEt and Ac-Tyr-OEt as well as the simple alkyl ester nucleophiles were purchased form Bachem (Switzerland). α-Bet-Phe-OEt was a gift from Prof. Dr. Wandrey, Institute of Biotechnology 2, Julich (Germany). Carboxamidomethyl(Cam) esters were synthesized by reaction of Boc-Phe-OH with iodacetamide in the presence of dicyclohexylamine in dry dioxan as described elsewhere[8b]. The dipeptide derivative was prepared via mixed anhydride coupling. The free N-terminus was obtained by cleaving the Boc-protecting group with trifluoracetic acid.

Peptide synthesis in frozen aqueous system. Preparative synthesis reaction in a total reaction volume of 27 ml (divided into nine portions of each 3 ml) were carried out in a cryostat F3 (Haake,Germany) using 5 ml polypropylene tubes (Nalgene). In order to achieve rapid freezing in liquid nitrogen all tubes and solutions should be precooled in a refrigerator (-10°C). This avoids ester hydrolysis after enzyme addition and mixing the reaction components before the solutions are put into nitrogen. The reactions were performed in 0.2 M sodium carbonate-bicarbonate buffer pH 10 containing 0.2 M NaCl, which is critical to achieve high yields. For α-chymotrypsin-catalyzed syntheses a reaction temperature of -25°C and for trypsin- and papain-catalyzed reactions -15°C was applied. Stock solutions of the enzymes were as follows: chymotrypsin/trypsin in 1 mM HCl containing 10 mM $CaCl_2$; papain in 5 mM dithioerithritol. The substrate was dissolved in 13.5 ml of water

whereas the nucleophilic component was dissolved in 13.5 ml of the double concentrated buffer solution. The pH value of the nucleophile solution in the buffer was adjusted to pH 10 by deprotonating the hydrochloride with 2N NaOH. After mixing the precooled solutions and addition of the enzyme the tubes were placed immediately into liquid nitrogen for 2 min to achieve shock-freezing. Then the tubes were transfered from liquid nitrogen into the cryostat at the respective reaction temperature. After a reaction time of 48 h the reactions were stopped by adding 1.5 ml of 8% trifluoroacetic acid in water to each tube. Subsequently, the solutions were combined and concentrated on a vacuum evaporator.

HPLC purification. After analytical runs on a Thermo-Separation-Products system on a Vydac 218HS54 C-18 column (The Separations Group) preparative HPLC was performed on a Vydac 201HS1022 column (2.2 x 25 cm) with the eluants: (A) 5 % CH_3CN in 0.1 % TFA and (B) 0.1 % TFA 95 % CH_3CN. A gradient of 0-65 % (B) in 45 min and 65-100 % (B) in 5 min at a flow rate of 15 ml/min was used for separation. The product containing fractions were pooled, the acetonitrile evaporated and the remaining aqueous solution lyophilized.

α-Bet-Phe-Arg-OEt.2TFA: $C_{26}H_{39}ClF_6N_6O_8$ (713.1) calcd. C 43.79 H 5.51 N 11.79; found C 43.66 H 5.04 N 11.28, m/z $[M+H]^+$ 451, ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm): 1.18 (t, 3H, O-CH_2-C<u>H</u>$_3$); 1.51-1.81 (m, 2H, CγH_2, Arg); 2.70-2.76 (m, 2H, C$_{\beta,\delta}H_2$, Arg); 3.04 (m, 9H, $(CH_3)_3N^+$); 3.11 (m, 2H, C$_\beta H_2$, Phe); 4.02 (2t, 2H, C$_\alpha$H, Phe/ C$_\alpha$H, Arg); 4.06 (m, 2H, O-C<u>H</u>$_2$-CH_3); 4.25 (m, 2H, CH_2CO); 7.20-7.28 (m, 4H, arom Phe H(2,3)); 7.77 (m, 1H, arom Phe H(4)); 8.66 (d, 1H, NH, Arg); 8.86 (d, 1H, NH, Phe).

α-Bet-Phe-Arg-OPr: m/z $[M+H-Cl]^+$ 465, ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm): 1.18 (t, 3H, O-CH_2-CH_2-C<u>H</u>$_3$); 1.57-1.60 (m, 2H, CγH_2, Arg); 1.75 (m, 2H, O-CH_2-C<u>H</u>$_2$-CH_3) 2.40-2.49 (m, 2H, C$_{\beta,\delta}H_2$, Arg); 2.85 (m, 9H, $(CH_3)_3N^+$); 3.09 (m, 2H, C$_\beta H_2$, Phe); 4.11 (2t, 2H, C$_\alpha$H, Phe/ C$_\alpha$H, Arg); 4.23 (m, 2H, O-C<u>H</u>$_2$-CH_2-CH_3); 4.44 (m, 2H, CH_2CO); 6.64 (d, 2H, arom Phe H(2,6)); 7.04 (d, 4H, arom Phe H(3,5)); 7.46 (dd, 1H, arom Phe H(4)); 8.01 (d, 1H, NH, Arg); 8.36 (d, 1H, NH, Phe).

Ac-Tyr-Arg-OEt: m/z $[M+H]^+$ 408, ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm): 0.91 (t, 3H, O-CH_2-C<u>H</u>$_3$); 1.16 (m, 2H, CγH_2, Arg); 1.75 (s, 3H, CH_3-CO) 2.74 (m, 2H, C$_{\beta,\delta}H_2$, Arg); 2.96 (m, 2H, C$_\beta H_2$, Tyr); 4.06 (m, 2H, O-C<u>H</u>$_2$-CH_3); 6.65 (d, 2H, arom Tyr H(2)); 7.03 (d, 2H, arom Tyr H(3)); 7.18-7.29 (m, 5H, N$_{\omega,\omega'}H_2$/N$_\delta$H, Arg); 7.99 (d, 1H, NH, Arg); 8.36 (d, 1H, NH, Tyr); 9.14 (s, 1H, OH, Tyr).

Ac-Tyr-Arg-OPr.2TFA: $C_{24}H_{33}F_6N_5O_9$ (649.5) calcd. C 44.38 H 5.12 N 10.78; found C 44.18 H 6.08 N 11.20, m/z $[M+H]^+$ 422, ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm):): 1.25 (t, 3H, O-CH_2-CH_2-C<u>H</u>$_3$); 1.51 (m, 4H, CγH_2, Arg/O-CH_2-C<u>H</u>$_2$-CH_3); 1.75 (s, 3H, CH_3-CO) 2.85/2.89 (m, 2H, C$_{\beta,\delta}H_2$, Arg); 3.11 (t, 2H, C$_\beta H_2$, Tyr); 4.08 (m, 4H, O-C<u>H</u>$_2$-CH_2-CH_3/C$_\alpha$H, Tyr, Arg); 6.64 (d, 2H, arom Tyr H(2)); 7.04 (d, 2H, arom Tyr H(3)); 7.28-7.48 (m, 5H, N$_{\omega,\omega'}H_2$/N$_\delta$H, Arg); 7.99 (d, 1H, NH, Arg); 8.36 (d, 1H, NH, Tyr); 9.14 (s, 1H, OH, Tyr).

α-Bet-Phe-Lys-OEt.2TFA: $C_{26}H_{39}ClF_6N_4O_8$ (685.1) calcd. C 45.59 H 5.74 N 8.18; found C 46.07 H 6.02 N 9.26, m/z $[M+H-Cl]^+$ 422, ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm): 1.25 (t, 3H, O-CH_2-$C\underline{H}_3$); 1.57-1.79 (m, 8H, $C_{\beta-\varepsilon}H_2$, Lys); 3.04 (m, 9H, $(CH_3)_3N^+$); 3.16 (m, 2H, $C_\beta H_2$, Phe); 4.07 (2t, 2H, $C_\alpha H$, Phe/$C_\alpha H$, Lys); 4.13 (m, 2H, O-$C\underline{H}_2$-CH_3); 4.20 (m, 2H, CH_2CO); 7.21-7.29 (m, 4H, arom Phe H(2,3)); 7.83 (m, 1H, arom Phe H(4)); 8.65 (d, 1H, NH, Lys); 8.89 (d, 1H, NH, Phe).

Ac-Tyr-Lys-OEt.TFA.H_2O: $C_{21}H_{32}F_3N_3O_8$ (511.5) calcd. C 49.31 H 6.31 N 8.22; found C 48.71 H 5.98 N 8.53, m/z $[M+H]^+$ 381, ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm): 1.23 (t, 3H, O-CH_2-$C\underline{H}_3$); 1.33-1.39 (m, 8H, $C_{\beta-\varepsilon}H_2$, Lys); 1.75 (s, 3H, CH_3-CO) 2.88 (m, 2H, $C_\beta H_2$, Tyr); 4.23 (q, 2H, O-$C\underline{H}_2$-CH_3); 4.42-4.44 (2t, 2H, $C_\alpha H$, Tyr/$C_\alpha H$, Lys); 6.63 (d, 2H, arom Tyr H(2)); 7.05 (d, 2H, arom Tyr H(3)); 7.67 (s, 2H, $N_\varepsilon H_2$, Lys) 8.32 (d, 1H, NH, Lys); 8.38 (d, 1H, NH, Tyr); 9.14 (s, 1H, OH, Tyr).

Bz-Arg-Phe-OMe.2TFA: $C_{27}H_{31}F_6N_5O_8$ (667.6) calcd. C 48.57 H 4.68 N 10.49; found C 48.74 H 5.59 N 11.69, m.p. 88-90°C, m/z $[M+H]^+$ 440, ^{1}H-NMR (300 MHz, DMSO-d_6, δ in ppm): 1.48-1.76 (m, 4H, $C_{\beta,\gamma}H_2$, Arg); 2.96-3.02 (m, 2H, $C_\beta H_2$, Phe); 3.09-3.32 (m, 2H, $C_\delta H_2$, Arg); 3.57 (s, 3H, O-CH_3); 4.48-4.51 (m, 2H, $C_\alpha H$, Phe/ $C_\alpha H$, Arg); 7.19-7.55 (m, 10H, arom Phe/Bz); 7.85 (d, 1H, NH, Arg); 8.38 (d, 1H, NH, Phe).

Bz-Arg-Phe-OCam.2TFA: $C_{28}H_{32}F_6N_6O_9$ (678.5) calcd. C 49.55 H 4.76 N 12.38; found C 47.98 H 5.87 N 12.94, m.p. 100-102°C, m/z $[M+H]^+$ 483, ^{1}H-NMR (300 MHz, DMSO-d_6, δ in ppm): 1.44-1.75 (m, 4H, $C_{\beta,\gamma}H_2$, Arg); 2.94-3.02 (m, 2H, $C_\beta H_2$, Phe); 3.05-3.19 (m, 2H, $C_\delta H_2$, Arg); 4.36-4.64 (m, 4H, $C_\alpha H$, Phe/ $C_\alpha H$, Arg, O-CH_2-CO); 7.18-7.55 (m, 12H, arom Phe/Bz, NH_2); 7.85 (d, 1H, NH, Arg); 8.42 (d, 1H, NH, Phe).

Bz-Arg-Ala-Phe-OCam.2TFA: m/z $[M+H]^+$ 554, ^{1}H-NMR (300 MHz, DMSO-d_6, δ in ppm): 1.19 (d, 3H, $C_\beta H_3$, Ala); 1.49-1.77 (m, 4H, $C_{\beta,\gamma}H_2$, Arg); 2.91-2.99 (m, 2H, $C_\beta H_2$, Phe); 3.07-3.17 (m, 2H, $C_\delta H_2$, Arg); 4.27-4.57 (m, 5H, $C_\alpha H$, Ala/ $C_\alpha H$, Phe/ $C_\alpha H$, Arg, O-CH_2-CO); 7.22-7.55 (m, 12H, arom Phe/Bz, NH_2); 7.86 (d, 1H, NH, Arg); 8.02 (d, 1H, NH, Ala); 8.41 (dd, 1H, NH, Phe).

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