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Enzymatic Synthesis of *C*-Terminal Arylamides of Amino Acids and Peptides

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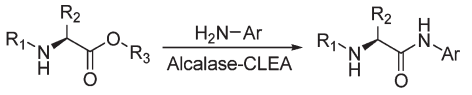

R_1 = carbamate protective group or peptide moiety
R_2 = amino acid side chain
R_3 = H, Me or Bn
Ar = aryl moiety

A mild and cost-efficient chemo-enzymatic method for the synthesis of *C*-terminal arylamides of amino acid and peptides is described. Using the industrial serine protease Alcalase under near-anhydrous conditions, *C*-terminal arylamides of *N*-Cbz-protected amino acids and peptides could be obtained from the corresponding *C*-terminal carboxylic acids, methyl (Me) or benzyl (Bn) esters, in high chemical and enantio- and diastereomeric purities. Yields ranged between 50% and 95% depending on the size of the aryl substituents and the presence of electron-withdrawing substituents. Complete α-*C*-terminal selectivity could be obtained even in the presence of various unprotected side-chain functionalities such as *β*/*γ*-carboxyl, hydroxyl, and guanidino groups. In addition, the use of the cysteine protease papain and the lipase Cal-B gave anilides in high yields. The chemo-enzymatic synthesis of arylamides proved to be completely free of racemization, in contrast to the state-of-the-art chemical methods.

Introduction

Arylamides, a class of carboxamides, are used in a wide variety of applications such as in polymers, peptidomimetics, dendrimers, scaffolds, and inhibitors and as cell-signaling molecules.[1] A special class is formed by the *C*-terminal amino acid or peptide arylamides, which are often used as substrates in chromogenic, fluorogenic or amperogenic enzymatic assays.[2] For this application, the arylamides should be optically pure. *p*-Nitroaniline (*p*NA, absorption at 405 nm) is often used for chromogenic assays and 7-amino-4-methylcoumarin (AMC, emission at 518 nm) for fluorogenic assays. For instance, over 1000 *C*-terminal peptide *p*NA and AMC arylamide substrates have been used for the determination of the activity of enzymes involved in blood coagulation.[3] Chemical methods for their preparation have

(1) (a) Vemparala, S.; Ivanov, I.; Pophristic, V.; Spiegel, K.; Klein, M. L. *Org. Lett.* **2006**, *27*, 693. (b) Pophristic, V.; Vemparala, S.; Ivanov, I.; Liu, Z.; Klein, M. L.; DeGrado, W. F. *J. Phys. Chem. B.* **2006**, *110*, 3517. (c) Yin, H.; Frederick, K. K.; Liu, D.; Wand, A. J.; DeGrado, W. F. *Org. Lett.* **2006**, *8*, 223. (d) Vinogradov, S. A. *Org. Lett.* **2005**, *7*, 1761. (e) Yin, H.; Gerlach, L. O.; Miller, M. W.; Moore, D. T.; Liu, D.; Vilaire, G.; Bennett, J. S.; DeGrado, W. F. *Bioorg. Med. Chem.* **2006**, *16*, 3380. (f) Meegalla, S. K.; Wall, M. J.; Chen, J.; Wilson, K. J.; Ballentine, S. K.; Desjarlais, R. L.; Schubert, C.; Crysler, C. S.; Chen, Y.; Molloy, C. J.; Chaikin, M. A.; Manthey, C. L.; Player, M. R.; Tomczuk, B. E.; Illig, C. R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3632. (g) Kirkland, T. A.; Adler, M.; Bauman, J. G.; Chen, M.; Haeggström, J. Z.; King, B.; Kochanny, M. J.; Liang, A. M.; Mendoza, L.; Phillips, G. B.; Thunnissen, M.; Trinh, L.; Whitlow, M.; Ye, B.; Ye, H.; Parkinson, J.; Guilford, W. J. *Bioorg. Med. Chem.* **2008**, *16*, 4963. (h) Foss, F. W. Jr; Snyder, A. H.; Davis, M. D.; Rouse, M.; Okusa, M. D.; Lynch, K. R.; MacDonald, T. L. *Bioorg. Med. Chem.* **2007**, *15*, 663.

(2) (a) Kembhavi, A. A.; Buttle, D. J.; Knight, C. G.; Barrett, A. J. *Arch. Biochem. Biophys.* **1993**, *303*, 208. (b) Stepanov, V. M.; Strongin, A. Y.; Izotova, L. S.; Abramov, Z. T.; Lyublinskaya, L. A.; Ermakova, L. M.; Baratova, L. A.; Belyanova, L. P. *Biochem. Biophys. Res. Commun.* **1977**, *77*, 298. (c) Clavin, S. A.; Bobbitt, J. L.; Shuman, R. T.; Smithwick, E. L. Jr. *Anal. Biochem.* **1977**, *80*, 355. (d) Talanian, R. V.; Quinlan, C.; Trautz, S.; Hackett, M. C.; Mankovich, J. A.; Banach, D.; Ghayur, T.; Brady, K. D.; Wong, W. W. *J. Biol. Chem.* **1997**, *272*, 9677. (e) Ulfohn, A.; Kramer, S. P.; Calle, S.; Sass, S.; Williamson, C. E.; Witten, B.; Seligman, A. M. *J. Surg. Res.* **1968**, *8*, 253.

(3) (a) Budzynski, A. Z. *Laboratory Medicine* **2001**, *32*, 365. (b) Hemker, H. C. *Handbook of Synthetic Substrates for the Coagulation and Fibrinolytic Systems*; Martinus Nijhoff Publishers: Boston, 1983.

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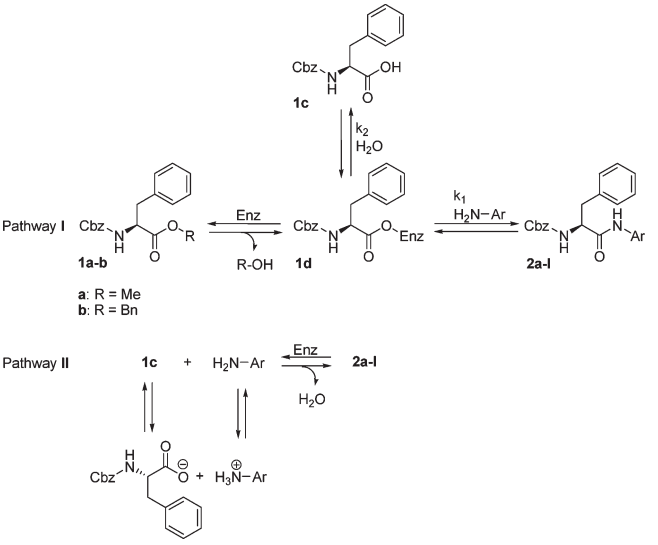
been investigated for decades and require highly reactive carboxylic acid derivatives for reaction with a free arylamine, due to its weak nucleophilicity. For example, the preparation of *N*-protected amino acid *p*NA derivatives required the use of $POCl_3$ in pyridine.[4] Often, most conventional coupling strategies, e.g., the DCC (*N*,*N*′-dicyclohexylcarbodiimide) method, do not result in satisfying yields.[5] Very commonly used in the past was the mixed anhydride method, which gave higher yields but also a high degree of racemization of the *C*-terminal amino acid residue.[6] This racemization could only be partially suppressed by the addition of copper(II) chloride.[7] Other methods based on reactive condensing agents, i.e., reactive phosphorus or boron derivatives, also led to significant racemization.[8] It appeared especially difficult to couple electron-deficient arylamines, such as those bearing a halogen, nitro, or cyano substituent. Very recently, a three-step protocol for the preparation of amino acid and peptide *C*-terminal electron-deficient arylamides was described, based on the reaction of an azide with a selenocarboxylate.[9] Although high yields were obtained, some racemization proved to be inevitable. Evidently, each of the known chemical methods for the preparation of *C*-terminal arylamides of amino acid and peptides suffers from some drawbacks. Besides the low yields and the racemization issue, the condensing reagents used are often hazardous, expensive, and consumed in stoichiometric amounts, leading to significant amounts of waste. Additionally, all reactive amino acid side chain functionalities have to be protected to prevent side reactions.

Compared to chemical approaches, chemo-enzymatic syntheses of arylamides may have several advantages such as the use of mild conditions, high selectivity, and complete absence of racemization. Moreover, the coupling reactions are catalyzed by (often recyclable) enzymes, and no stoichiometric amounts of expensive coupling reagents are used.[10] Furthermore, no amino acid side chain protection is needed.

There are two approaches in chemo-enzymatic amide synthesis, i.e., the kinetically controlled pathway and the thermodynamically controlled pathway (pathways **I** and **II**, respectively, in Scheme 1).[11]

Pathway **I**, starting from an α-amino ester, is usually preferred in chemo-enzymatic amide synthesis because the energy barrier for product formation is low. Reaction of the amine with the acyl−enzyme intermediate (**1d**) leads to the desired amide. However, after formation of **1d**, water can act as a nucleophile leading to undesired hydrolysis, so ideally $k_1 \gg k_2$. Nevertheless, during the course of the amidation, the amide product is hydrolyzed to the free carboxylic compound. Hence, there is an optimum in the amount of product and for a high product yield the reaction should be stopped after this optimum has been reached.

SCHEME 1



In the thermodynamically controlled pathway **II**, the energy barrier is high and the equilibrium is usually on the side of the starting materials. Furthermore, reaction rates are generally low due to the equilibrium between the protonated and (unreactive) deprotonated carboxylic acid. Manipulation of the reaction conditions, e.g., by crystallization of the final product during the amidation, is essential to obtain acceptable arylamide yields.

The thermodynamically controlled chemo-enzymatic synthesis of *C*-terminal α-amino arylamides was disclosed in the late 1930s.[12] High yields were obtained using proteases in buffered aqueous systems due to rapid precipitation of the products. However, this method is limited to relatively strong arylamine nucleophiles such as aniline, and hydrophobic amino acids are required to obtain precipitation of the product. Furthermore, in the case that peptides are *C*-terminally arylamidated, simultaneous hydrolysis of the peptide bonds occurs. Kato et al. describe[13] the kinetically controlled chemo-enzymatic arylamidation with the less nucleophilic *p*NA using Alcalase in organic solvents with a water content of 5%; however, their reported yields do not exceed 25%.

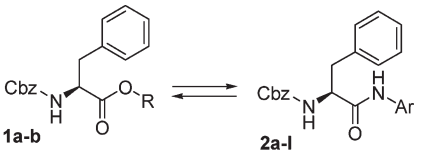
Herein, we report a versatile, mild, high-yielding, selective, and racemization-free chemo-enzymatic strategy for the synthesis of amino acid and peptide *C*-terminal arylamides either kinetically (**I**) or thermodynamically (**II**) using the protease Alcalase or the lipase Cal-B in organic solvents or the protease papain in two-phase systems.

Results and Discussion

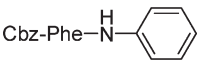
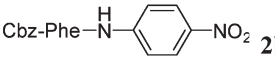
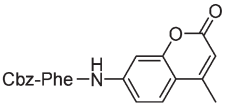
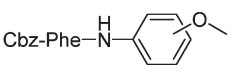
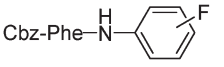
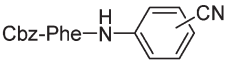
Alcalase,[14] a cheap industrial serine protease from *Bacillus licheniformis*, has been extensively used for various hydrolytic

(4) Rijkers, D. T. S.; Adams, H. P. H. M.; Hemker, H. C.; Tesser, G. I. *Tetrahedron* **1995**, *51*, 11235.
(5) Zimmerman, M.; Yurewicz, E.; Patel, G. *Anal. Biochem.* **1976**, *70*, 258.
(6) Benoiton, N. L.; Lee, Y. C.; Steinaur, R.; Chen, F. M. *Int. J. Pept. Protein Res.* **1992**, *40*, 559.
(7) Houben-Weyl, Methods of Organic Chemistry. *Synthesis of Peptides and Peptidomimetics*, 4th ed.; Thieme Verlag: Stuttgart, 2002; Vol. E22a, p 586.
(8) Kato, T.; Nagatsu, T.; Kimura, T. *Experientia* **1978**, *34*, 319.
(9) Wu, X.; Hu, L. *J. Org. Chem.* **2007**, *72*, 765.
(10) Guzmán, F.; Barberis, S.; Illanes, A. *Electr. J. Biotech.* **2007**, *10*, 279.
(11) Bordusa, F. *Braz. J. Med. Biol. Res.* **2000**, *33*, 469.
(12) (a) Bergmann, M.; Behrens, O. K. *J. Biol. Chem.* **1938**, *124*, 7. (b) Bergmann, M.; Fruton, J. S. *J. Biol. Chem.* **1938**, *124*, 321. (c) Behrens, O. K.; Doherty, D. G.; Bergmann, M. *J. Biol. Chem.* **1940**, *136*, 61. (d) Dikker, C. A.; Fruton, J. S. *J. Biol. Chem.* **1948**, *173*, 471. (e) Hanson, H. T.; Smits, E. L. *J. Biol. Chem.* **1949**, *179*, 115. (f) Doherty, D. G.; Popenoe, E. A. *J. Biol. Chem.* **1951**, *189*, 447.
(13) Kato, Y.; Asano, Y.; Nakazawa, A.; Kondo, K. *Tetrahedron* **1989**, *45*, 5743.
(14) Available from Novozymes (Bagsvaerd, Denmark).

TABLE 1. Yields and Enantiomeric Excess (ee) of Synthesized Arylamides Using Alcalase-CLEA as Biocatalyst[a]



a: R = Me
b: R = Bn

Entry	Precursor		equiv. arylamine	Amide product		Yield, (%)[b]	e.e. (%)[c], (%)[d]
1	Cbz-Phe-OMe	(**1a**)			**2a**	93	n.d.[e]
2	Cbz-Phe-OBn	(**1b**)	10		**2a**	94	n.d.
3	Cbz -Phe-OH	(**1c**)			**2a**	93	>99.5, 98.9
4[f]	Cbz -Phe-OH	(**1c**)	11	 (*p*NA)	**2b**	65	>99.5, 96.2
5	Cbz -Phe-OH	(**1c**)	9	 (AMC)	**2c**	51	>99.5, 95.7
6					**2d** (*o*)	87	n.d.
7	Cbz -Phe-OH	(**1c**)	12		**2e** (*m*)	86	n.d.
8					**2f** (*p*)	88	n.d.
9					**2g** (*o*)	85	n.d.
10	Cbz -Phe-OH	(**1c**)	14		**2h**(*m*)	79	n.d.
11					**2i** (*p*)	81	n.d.
12					**2j** (*o*)	70	>99.5, 97.6
13	Cbz -Phe-OH	(**1c**)	13		**2k**(*m*)	84	>99.5, 97.7
14					**2l** (*p*)	65	>99.5, 97.2

[a] All reactions were performed in MTBE (methyl *tert*-butyl ether, except entry 4) at 50 °C and stopped after 16 h. [b] Isolated yields based on the acyl donor. [c] Of enzymatically synthesized arylamide. [d] Of chemically synthesized arylamide following the literature procedure.[19] [e] Not determined. [f] Reaction was performed in THF (tetrahydrofuran).

reactions and condensations. This biocatalyst displays a relatively broad substrate tolerance and has been used in kinetically controlled enzymatic peptide synthesis.[15] High yields are usually obtained due to the stability of the enzyme in nearly anhydrous (water contents <1 wt %) organic solvents, thereby minimizing product and/or ester hydrolysis.[16] Therefore, we selected Alcalase in a first attempt to synthesize arylamides via the kinetically controlled pathway using Cbz-Phe-OMe (**1a**) as model substrate and aniline as arylamine.

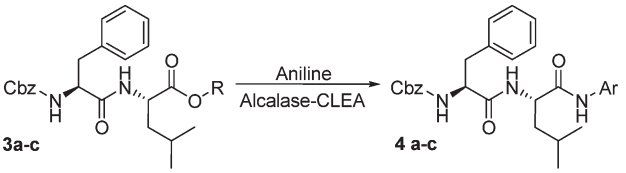
When Alcalase (commercially available as 10 wt % aqueous solution) was isolated and dried by precipitation with *t*-BuOH as described by Chen et al.[17] followed by addition to Cbz-Phe-OMe (**1a**) in pure aniline, the yield of arylamide **2a** did not exceed 27% (based on HPLC analysis). An equilibrium mixture of arylamide **2a** and hydrolyzed starting material Cbz-Phe-OH (**1c**) was observed, and the water content proved to be critical for the yield of the arylamide. Surprisingly, the addition of 4 Å molecular sieves resulted in almost complete (>95%, HPLC analysis) conversion to arylamide **2a** with the enzyme still remaining active. The increased yield may be explained by the fact that not only water but also MeOH is trapped by 4 Å molecular sieves. After workup, NMR analysis showed contaminations derived from the

(15) (a) Maurer, K. H. *Curr. Opin. Biotechnol.* **2004**, *15*, 330. (b) Smith, E. L.; DeLange, R. J.; Evans, W. H.; Landon, M.; Markland, F. S. *J. Biol. Chem.* **1968**, *243*, 2184. (c) Siezen, R. J.; Leunissen, J. *J. Protein Sci.* **1997**, *6*, 501.

(16) Wang, P.; Hill, T. G.; Wartchow, C. A.; Huston, M. E.; Oehler, L. M.; Smith, M. B.; Bednarski, M. D.; Callstrom, M. R. *J. Am. Chem. Soc.* **1992**, *114*, 378.

(17) Chen, S. -T.; Chen, S. -Y.; Wang, K. -T. *J. Org. Chem.* **1992**, *57*, 6960.

TABLE 2. Yields of Dipeptide Arylamides and ee's of the Amino Acid Residues[a]



entry	precursor		amide product	yield[b] (%)	ee[c,d] (%)
1	Cbz-Phe-Leu-OMe	**3a**	Cbz-Phe-Leu-anilide	93	n.d.[e]
2	Cbz-Phe-Leu-OBn	**3b**	Cbz-Phe-Leu-anilide	94	n.d.
3	Cbz-Phe-Leu-OH	**3c**	Cbz-Phe-Leu-anilide	93	>99.5[f], 95.7[f] >99.5[g], 96.8[g]
4	Cbz-Phe-Leu-OH	**3c**	Cbz-Phe-Leu-*p*NA	83	n.d.
5	Cbz-Phe-Leu-OH	**3c**	Cbz-Phe-Leu-AMC	77	n.d.

[a] All reactions were performed in MTBE at 50 °C using 10 equiv of aniline and stopped after 16 h. [b] Isolated yields based on the acyl donor. [c] Of enzymatically synthesized arylamide. [d] Of chemically synthesized[19] arylamide. [e] Not determined. [f] Phe moiety. [g] Leu moiety.

Alcalase solution. When Alcalase cross-linked enzyme aggregates (CLEA)[18] were used, equally high yields of **2a** could be obtained starting from **1a** or benzyl ester **1b** (Table 1, entries 1 and 2), without any contaminations in the final product. Sufficiently dry Alcalase-CLEA was obtained by washing once with *t*-BuOH. After the reaction it was conveniently removed by simple filtration.

In subsequent amidations, the amount of arylamine was decreased using the cosolvents toluene, THF, or MTBE. To our surprise, the thermodynamic approach (**II**) using the free carboxylic acid Cbz-Phe-OH (**1c**) and anhydrous aniline in the presence of 3 Å molecular sieves gave equally high yields of the arylamide **2a** (Table 1, entry 3). In addition, reaction times were comparable to those starting from an amino acid ester (entries 1 and 2). In order to broaden the scope of these amidation reactions, we investigated whether the weakly nucleophilic *p*NA ($pK_a = 1.0$, vs $pK_a = 5.2$ for aniline), the sterically demanding AMC, as well as a number of methoxy-, fluoro-, and cyano-substituted anilines could be used in the Alcalase-mediated arylamidations (see Table 1, entries 4−14). To our satisfaction, high yields of arylamides (**2b**−**l**) were obtained although the efficiency of *p*NA, and AMC was clearly lower than that of aniline. To verify the absence of racemization during the enzymatic arylamidation, ee's were determined by chiral HPLC and compared to chemically synthesized arylamides (see Table 1).[19] As expected, no detectable racemization was observed using the enzymatic reactions, in sharp contrast to the conventional chemical arylamidations using PCl_3 in pyridine, where significant racemization is observed (1−5%).

At this point, it was important to address some industrially relevant aspects of these enzymatic conversions. First, we proved that, in order to obtain the required anhydrous conditions, the use of molecular sieves, which are unamenable for use at large scale, could be replaced by azeotropic distillation. For instance, the conversion of **1c** to **2a** using 1:1 (v/v) aniline/toluene proceeded to >95% when toluene was continuously added and evaporated at reduced pressure. Second, we demonstrated that Alcalase-CLEA, after conversion of **1c** to **2a**, could be easily separated from the reaction mixture by a rapid filtration and washing step.

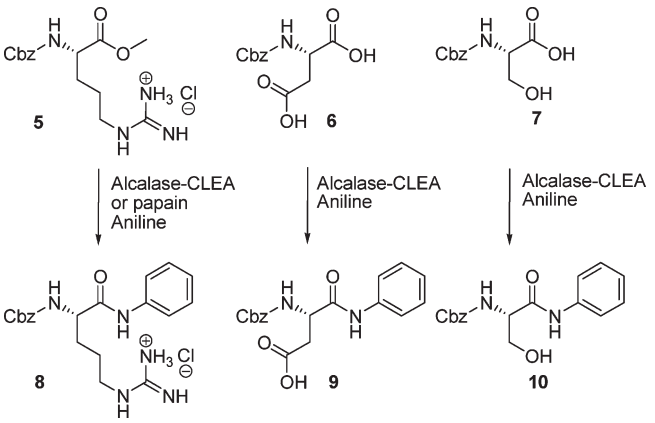
Of even greater interest than of amino acids are *C*-terminal arylamides of peptides since they are pre-eminently used as substrates for enzymatic assays. Using both the thermodynamic (**II**) and the kinetic (**I**) approach, dipeptides **3a**−**c** were smoothly converted into the corresponding *C*-terminal anilides **4a**−**c** (Table 2, entries 1−5, 77−94% yield). No side products were detected by HPLC indicating that no peptidic bond hydrolysis nor transamidation had occurred in all cases. In addition, no detectable racemization of the Leu residue had taken place as was demonstrated by peptide hydrolysis followed by chiral HPLC of the resulting amino acids. This is in sharp contrast to the corresponding chemical conversions[19] (using PCl_3) were inevitable racemization occurs leading to ee values of 96% of each residue in the dipeptide.

Besides the absence of racemization, another advantage of the enzymatic arylamidation is that no protection of the amino acid side chains is required. This is again in contrast to chemical coupling methods where reactive side chain functionalities such as the β- or γ-carboxyl group of aspartic and glutamic acid, the hydroxyl function of serine, threonine or tyrosine and the guanidino function of arginine need protection. We recently demonstrated that Alcalase-CLEA can be used for the regioselective esterification[20] and hydrolysis[21] of Asp and Glu derivatives. Now we illustrate this advantage by the smooth and regioselective conversion of Cbz-Arg-OMe **5**, Cbz-Asp-OH **6**, and Cbz-Ser-OH **7** to the corresponding *C*-terminal anilides **8**, **9**, and **10** (Table 3, entries 1−4).

Very high yields of anilides **9** and **10** (Table 3, entries 3 and 4, 91−93%) were obtained using Alcalase-CLEA. For Cbz-Asp-OH (**6**), the α-*C*-terminal selectivity appeared to be 100% as proven by comparison (HPLC and NMR) with the chemically synthesized reference compound. In the

(18) Alcalase-CLEA, Codexis, 650 AGEU/g; see also: Sheldon, R. A. *Biochem. Soc. Trans.* **2007**, *35*, 1583.
(19) Oyamada, H.; Saito, T.; Inaba, S.; Ueki, M. *Chem. Soc. Jpn* **1991**, *64*, 1422.
(20) Nuijens, T.; Cusan, C.; Kruijtzer, J. A. W.; Rijkers, D. T. S.; Liskamp, R. M. J.; Quaedflieg, P. J. L. M. *Synthesis* **2009**, *5*, 809.
(21) Nuijens, T.; Cusan, C.; Kruijtzer, J. A. W.; Rijkers, D. T. S.; Liskamp, R. M. J.; Quaedflieg, P. J. L. M. *Tetrahedron Lett.* **2009**, *50*, 2719.

TABLE 3. Yields of Synthesized Arylamides of Cbz-Arg-OMe, Cbz-Asp-OH, and Cbz-Ser-OH[a]



entry	precursor	enzyme	product	yield (%)
1	Cbz-Arg-OMe **5**	Alcalase-CLEA	**8**	54
2	Cbz-Arg-OMe **5**	papain	**8**	82
3	Cbz-Asp-OH **6**	Alcalase-CLEA	**9**	91
4	Cbz-Ser-OH **7**	Alcalase-CLEA	**10**	93

[a] Reactions were performed in THF (entries 1 and 2) or MTBE (entries 3 and 4) at 50 °C using 10 equiv of aniline and were stopped after 16 h. Isolated yields are based on the acyl donor. Cbz-Arg-OMe was used instead of Cbz-Arg-OH due to solubility problems with the latter in anhydrous organic solvents.

TABLE 4. Yields of Synthesized Cbz-Ala Arylamide Using Cal-B[a]

entry	precursor		product	yield[b] (%)	ee (%)
1	Cbz-Ala-OMe	**11a**	**12**	92	>99.5
2	Cbz-Ala−OH	**11b**	**12**	77	>99.5

[a] All reactions were performed in MTBE at 50 °C using 10 equiv of aniline and were stopped after 16 h. [b] Isolated yields based on the acyl donor.

arylamidation of Cbz-Ser-OH (**7**) only a small amount (2%) of the side chain ester was formed, as was demonstrated by LC-MS. However, Alcalase does not favor positively charged amino acids at the P_1 position[22] in the active site, resulting in only a moderate yield of **8** (Table 3, entry 1, 54%). To remedy this, the widely employed and commercially available cysteine protease papain was used, which does favor positively charged amino acids at its P_1 position. The application of this enzyme in pure aniline did not result in any conversion of **5**, since papain is not active in nearly anhydrous organic solvents. Therefore, a two-phase system consisting of aniline and phosphate buffer (pH = 7.5) was used, and a high yield of anilide **8** (Table 3, 82%) was readily obtained after only 10 min.

In order to broaden the scope of this technology beyond proteases, we also explored the use of lipases. We envisioned that *Candida antarctica* lipase-B (Cal-B) would be promising in our application since it has a broad substrate tolerance for aliphatic acids,[23] is known to be active in the transesterification of amino acids,[24] and maintains a high activity in anhydrous organic solvents.[25] Hence, we subjected Cbz-Ala derivatives **11a** and **11b** to Cal-B and aniline under anhydrous conditions. Indeed, the corresponding anilide **12** was obtained in high yield (Table 4, entries 1 and 2).

Conclusions

In this paper, we have shown that *N*-terminal-protected amino acids and peptides can be enzymatically converted in high yields into various *C*-arylamides under (nearly) anhydrous conditions even if the aryl amine has an extremely low nucleophilicity or is sterically very demanding. Although Alcalase readily accepted most *C*-terminal moieties in its active site, in some cases, for instance with *C*-terminal positively charged arginine residues, the use of papain in a two-phase system gave more satisfactory results. Starting from either *C*-terminal free carboxylic acids or *C*-terminal alkyl esters, high yields were obtained, and in case of dipeptides, no peptide bond hydrolysis or transamidation occurred. In contrast to the state-of-the-art chemical methods, no racemization occurred at the *C*-terminal position and no amino acid side chain protection was required. Thus, we feel that this enzymatic method is a significant step forward in the synthesis of this important class of compounds. Since the (nearly) anhydrous conditions can be obtained by (azeotropic) distillation, this technology is also amenable to scale-up.

Experimental Section

General Procedure for the Synthesis of Arylamides 2a and 4a. Alcalase−CLEA (500 mg) was added to a mixture containing MTBE (4.5 mL), aniline (500 μL, 4.48 mmol), **1a**−**c** or **3a**−**c** (0.45 mmol), and 3 Å molecular sieves (200 mg). The mixtures were shaken at 50 °C at 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter, and the solids were washed with ethyl acetate (EtOAc, 50 mL, 3×). Distilled water (150 mL) was added to the combined filtrates, and under vigorous stirring the pH was adjusted to 3.0 with 1 N aqueous HCl solution. After an additional 10 min of stirring the two layers were separated. This procedure of washing the organic layer with an aqueous phase of pH 3 was repeated twice. Subsequently, the organic phase was washed with saturated aqueous $NaHCO_3$ (3 × 100 mL) and brine (100 mL), dried (Na_2SO_4), and concentrated in vacuo. The residue was diluted with *n*-heptane (2 mL), and the resulting crystals were isolated by filtration and washed with *n*-heptane (2 × 5 mL).

Cbz-Phe-Leu-anilide (4a). Compound **4a** was obtained as a solid (from: **3a**, 93% yield, 204 mg; **3b**, 94% yield, 206 mg; **3c**, 93% yield, 204 mg): t_R (HPLC method 1) 21.08 min; purity 99%; R_f (EtOAc/*n*-hexane, 1/1, v/v) 0.52; mp 225 °C; $[\alpha]^{20}_D$ = 16.8 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, $CDCl_3$) δ = 0.81 (d, J = 6.0 Hz, 6 H), 1.34−1.50 (m, 2 H), 1.65−1.76 (m, 1 H), 3.00 (d, 2 H), 4.30−4.51 (m, 2 H), 5.00 (s, 2 H), 5.21 (d, J = 6.6 Hz, 1 H), 6.25 (d, J = 7.2 Hz, 1 H), 7.00−7.27 (m, 13 H), 7.47 (d, J = 7.5 Hz, 2 H), 8.25 (s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 21.7, 22.4, 24.5, 38.4, 41.0, 51.6, 51.9, 55.7, 66.6, 126.5, 127.6,

(22) Tai, D. -F. *Curr. Org. Chem.* **2003**, *7*, 515.

(23) Otto, R. T.; Scheib, H.; Bornscheuer, U. T.; Pleiss, J.; Syldatk, C.; Schmid, R. D. *J. Mol. Catal. B* **2000**, *8*, 201.

(24) Otto, R. T.; Scheib, H.; Bornscheuer, U. T.; Pleiss, J.; Syldatk, C.; Schmid, R. D. *Biotechnol. Lett.* **1992**, *14*, 789.

(25) Zoete, M. C.; Kock-van Dalen, A. C.; Rantwijk, F.; Sheldon, R. A. *J. Mol. Catal. B* **1996**, *2*, 19.

127.8, 128.2, 129.2, 136.1, 136.3, 155.9, 171.0, 172.7; FIA-ESI-(+)-TOF-MS m/z $[M + H]^+$ calcd for $C_{29}H_{34}N_3O_4$ 488.2543, found 488.2559; IR (neat/cm^{-1}) 3281, 1692, 1641, 1531, 1495, 1447, 1285, 1258, 1234.

General Procedure for the Synthesis of Arylamides 2b and 4b. Alcalase−CLEA (500 mg) was added to a mixture containing THF (5 mL), *p*NA (500 mg, 3.6 mmol), **1c** or **3c** (0.33 mmol), and 3 Å molecular sieves (200 mg). The mixture was shaken at 50 °C with 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter, and the solids were washed with EtOAc (50 mL, 3×). The combined filtrate was concentrated in vacuo and purified by preparative HPLC.

Cbz-Phe-Leu-*p*-nitroanilide (4b). Compound **4b** was obtained as a solid in 83% yield (145 mg): t_R (HPLC method 1) 20.93 minp purity 96%; R_f (EtOAc/n-hexane, 1/1, v/v) 0.60; mp 196 °C; $[\alpha]^{20}_D$ = 26.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 0.98 (dd, J = 6.3 and 10.2 Hz, 6 H), 1.55−1.80 (m, 3 H), 2.76−2.84 (dd, 1 H), 3.05−3.11 (dd, 1 H), 4.35−4.43 (m, 1 H), 4.51−4.59 (m, 1 H), 5.01 (s, 2 H), 7.20−7.54 (m, 10 H), 7.52 (d, J = 8.7 Hz, 1 H), 7.93 (d, J = 9.0 Hz, 2 H), 8.29 (d, J = 9.3 Hz, 1 H), 8.36 (d, J = 7.5 Hz, 1 H), 10.7 (s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 21.5, 22.8, 24.2, 37.2, 52.2, 55.7, 65.0, 118.9, 124.8, 126.1, 127.5, 127.8, 128.1, 129.1, 136.9, 137.8, 142.2, 144.9, 155.7, 171.6, 171.9; FIA-ESI(+)-TOF-MS m/z $[M + H]^+$ calcd for $C_{29}H_{33}N_4O_6$ 533.2395, found 533.2411; IR (neat/cm^{-1}) 3277, 2958, 1691, 1647, 1532, 1515, 1341, 1300, 1254.

General Procedure for the Synthesis of Arylamides 2c−l and 4c. Alcalase−CLEA (500 mg) was added to a mixture containing MTBE (4.5 mL), arylamine (500 mg), **1c** or **3c** (100 mg, 0.33 mmol), and 3 Å molecular sieves (200 mg). The mixture was shaken at 50 °C with 150 rpm for 16 h and subsequently filtered over a P4 glass filter. The solids were washed with EtOAc (50 mL, 3×). The combined filtrate was concentrated in vacuo, and the resulting residue was purified by preparative HPLC.

Cbz-Phe-2-methoxyanilide (2d). Compound **2d** was obtained in 87% yield as a solid (116 mg): t_R (HPLC method 1) 20.41 min; purity 99%; R_f (EtOAc/*n*-hexane, 1/1, v/v) 0.56; mp 160 °C; $[\alpha]^{20}_D$ = 86.2 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 2.85 (dd, 1 H), 3.12 (dd, 1 H), 3.81 (s, 3 H), 4.55 (m, 1 H), 4.98 (m, 2 H), 6.92 (m, 1 H), 7.20−7.40 (m, 12 H), 7.77 (d, J = 8.4 Hz, 1 H), 8.01 (m, 1 H), 9.24 (s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 37.0, 55.7, 56.7, 65.2, 111.1, 120.2, 121.0, 124.3, 126.2, 126.9, 127.3, 127.6, 127.9, 128.2, 129.2, 136.8, 137.8, 149.1, 155.9, 170.2; FIA-ESI(+)-TOF-MS m/z $[M+H]^+$ calcd for $C_{24}H_{25}N_2O_4$ 405.1808, found 405.1783; IR (neat/cm^{-1}) 3291, 1685, 1656, 1598, 1531, 1499, 1444, 1326, 1263, 1246, 1051.

Cbz-Arg-anilide (8). (a) Using Alcalase−CLEA. Alcalase−CLEA (500 mg) was added to a mixture containing THF (4.5 mL), aniline (500 μL, 4.5 mmol, 10 equiv), **5** (145 mg, 0.45 mmol, 1 equiv), and 3 Å molecular sieves (200 mg). The mixture was shaken at 50 °C with 150 rpm for 16 h and subsequently filtered over a P4 glass filter. The solids were washed with EtOAc (50 mL, 3×), and the combined filtrate was concentrated in vacuo. The residue was triturated in distilled water (20 mL), the resulting crystals were isolated by filtration over a P4 glass filter, washed with water (50 mL, 2×), and resuspended in toluene (50 mL), and the mixture was concentrated in vacuo.

(b) Using Papain. Papain (10 mg) was added to a mixture containing phosphate buffer (2.5 mL, 250 mM, pH 7.5), aniline (2.5 mL), 1,4-dithiothreitol (1 mg), and **5** (145 mg, 0.45 mmol). The mixture was shaken at ambient temperature for 10 min followed by the addition of EtOAc (100 mL) and distilled water (100 mL). Under vigorous stirring, the pH was adjusted to 3.0 with 1 N aqueous HCl solution. After an additional 10 min of stirring, the two layers were separated. This procedure of washing the organic layer with an aqueous phase of pH 3 was repeated twice. The organic phase was concentrated in vacuo, and the residue was suspended in distilled water (20 mL). The resulting crystals were isolated by filtration over a P4 glass filter, washed with distilled water (50 mL, 2×), and resuspended in toluene (100 mL), and the mixture was concentrated in vacuo.

Compound **8** was obtained as a hygroscopic solid: (a) 54% yield, 93 mg; (b) 82% yield, 141 mg; t_R(HPLC method 1) 9.46 min; purity 98%; R_f (EtOAc/*n*-hexane, 1/1, v/v) 0.31; mp 56 °C; $[\alpha]^{20}_D$ = 44.8 (*c* 0.5, DMSO); ^{1}H NMR (300 MHz, DMSO-d_6) δ = 1.40−1.90 (m, 4 H), 3.13 (m, 2 H), 4.20 (m, 1 H), 5.04 (s, 2 H), 7.00−7.48 (m, 12 H), 7.64−7.36 (m, 3 H), 7.93 (s, 1 H) 10.28 (s, 1 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ = 25.1, 28.8, 54.8, 65.3, 119.1, 123.2, 127.6, 127.7, 128.2, 128.5, 136.9, 138.9, 155.9, 156.9, 170.7; FIA-ESI(+)-TOF-MS m/z $[M + H]^+$ calcd for $C_{20}H_{26}N_5O_3$ 384.2030, found 384.2041; IR (neat/cm^{-1}) 3292, 3260, 1748, 1597, 1553, 1498, 1488, 1434, 1368, 1321, 1262.

Cbz-Asp-anilide (9).[26] Alcalase−CLEA (500 mg) was added to a mixture containing MTBE (4.5 mL), aniline (500 μL, 4.5 mmol, 10 equiv), Cbz-Asp-OH **6** (120 mg, 0.45 mmol, 1 equiv), and 3 Å molecular sieves (200 mg). The mixture was shaken at 50 °C with 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter, and the solids were washed with EtOAc (50 mL, 3×) and aqueous HCl (50 mL, pH = 1, 3×). The two phases of the filtrate were separated, and the organic layer was concentrated in vacuo. After preparative HPLC purification, compound **9** was obtained in 91% yield as a solid (140 mg).

Cbz-Ser-anilide (10).[27] Compound **10** was synthesized as described for **9** using Cbz-Ser-OH **7** (108 mg, 0.45 mmol, 1 equiv) and obtained in 93% yield as a solid (423 mg).

Cbz-Ala-anilide (12).[28] Cal-B (100 mg) was added to a mixture containing MTBE (4.5 mL), aniline (500 μL), **11a** or **11b** (100 mg), and molecular sieves (200 mg). The mixture was shaken at 50 °C at 150 rpm for 16 h. The reaction mixture was filtered over a P4 glass filter, and the solids were washed with EtOAc (50 mL, 3×). Distilled water (150 mL) was added to the filtrate, and under vigorous stirring the pH was adjusted to 3.0 with 1 N aqueous HCl solution. After an additional 10 min of stirring, the two layers were separated. This procedure of washing the organic layer with an aqueous phase of pH 3 was repeated twice. Subsequently, the organic phase was washed with saturated aqueous $NaHCO_3$ (100 mL, 2×) and brine (100 mL), dried (Na_2SO_4), and concentrated in vacuo. The residue was diluted with *n*-heptane (2 mL), and the resulting crystals were isolated by filtration and washed with *n*-heptane (2 × 5 mL) giving compound **12** (from: **11a**, 92% yield, 116 mg; from **11b**, 77% yield, 103 mg).

Supporting Information Available: Experimental procedures for the preparation of compounds **1a**,**b**, **3a**−**c**, **5**, **9**, and **11a**. ^{1}H NMR, ^{13}C NMR, HR-MS, and HPLC data for all synthesized compounds. Additional IR absorption spectra for compounds **2a**−**l**, **4a**−**c**, **6**, **9**, **10**, and **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

(26) Huang, X.; Luo, X.; Roupioz, Y.; Keillor, J. W. *J. Org. Chem.* **1997**, *62*, 8821.
(27) Adkins, H.; Ross, R. M.; Schroeder, D. C.; Mahoney, C. L.; Gilbert, W. W. *J. Am. Chem. Soc.* **1954**, *76*, 147.
(28) Kamiński, Z. J.; Kolesińska, B.; Kamińska, J. E.; Góra, J. *J. Org. Chem.* **2001**, *66*, 6276.