DOI: 10.1002/cbic.201200017

Papain-Specific Activating Esters in Aqueous Dipeptide Synthesis

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Enzymatic peptide synthesis has the potential to be a viable alternative for chemical peptide synthesis. Because of the increasing commercial interest in peptides, new and improved enzymatic synthesis methods are desirable. In recently developed enzymatic strategies such as substrate mimetic approaches and enzyme-specific activation, use of the guanidinophenyl ester (OGp) group has been shown to suffer from some drawbacks. OGp esters are sensitive to spontaneous chemical hydrolysis and the group is expensive to synthesize and therefore not suitable for large-scale applications. On the basis of earlier computational studies, we hypothesized that OGp might be replaceable by simpler ester groups to make the enzyme-specific activation approach to peptide bond formation more accessible. To this end, a set of potential activating esters (Z-Gly-Act) was designed, synthesized, and evaluated. Both the benzyl (OBn) and the dimethylaminophenyl (ODmap) esters gave promising results. For these esters, the scope of a model dipeptide synthesis reaction under aqueous conditions was investigated by varying the amino acid donor. The results were compared with those obtained from a previous study of Z-X_{AA}-OGp esters. Computational docking analysis of the set of esters was performed in order to provide insight into the differences in the reactivities of all the potential activating esters. Finally, selected ODmap- and OBn-activated amino acids were applied in the synthesis of two biologically active dipeptides on preparative scales.

Introduction

The market share of peptide-based drugs is steadily increasing and will likely continue to do so, because a large number of therapeutic peptides are in clinical trials.[1] Other relevant applications of peptides include their use as nutrients[2] and cosmetic ingredients.[3] Large-scale peptide synthesis, however, is an expensive and time-consuming procedure, and this conflicts with the considerable commercial interest.[4]

Solid-phase peptide synthesis (SPPS) is the most commonly used and most versatile strategy for the synthesis of peptides in general.[5] The requirement for side-chain protection, the need to use excesses of reagents, and the risk of racemization are downsides of this method, but are to a large extent compensated by its capability to incorporate virtually any amino acid. A strategy commonly used in cases of larger peptides is to produce shorter fragments by SPPS and to couple them in solution to make the synthesis more convergent.[6] Alternatively, fragments with unprotected side chains can be coupled in aqueous solution by the chemoselective native chemical ligation method, which involves a C-terminal thioester and an N-terminal cysteine.[7] For large-scale production of long peptides (>50 amino acid residues) and protein sequences containing only natural amino acids, fermentation is currently most feasible, but this requires significant development efforts for each individual peptide or protein.[8]

A relatively new and rather unexplored area is the use of enzymatic hydrolysis of readily available existing proteins to release specific peptides.[4a] An alternative use of these proteases, though, is their potential to *create* peptide bonds.[9] Advantages of such a chemoenzymatic approach include the high selectivities of the enzymes, minimal need for side chain protection, and absence of racemization. On the other hand, the generally narrow substrate specificities of enzymes, in particular the exclusion of unnatural amino acids, severely restricts their universal application. In addition, undesired product hydrolysis when using proteases can be problematic, even though strategies have been developed to minimize this side reaction.[9] By employing the principle of substrate mimetics,[10] the challenge of inadequate substrate recognition can be overcome. In the substrate mimetics approach, the amino acid side chain—crucial for enzyme recognition—is transferred to the ester leaving group so that the enzymatic process becomes independent of the amino acid to be coupled. The guanidinophenyl (OGp) group (Scheme 1, below), a mimetic of the arginine side chain, has been extensively applied in academic research with much

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success.[11] However, the OGp group is unsuitable for industrial application because it is expensive and difficult to work with due to the positively charged (under relevant conditions) guanidino group.

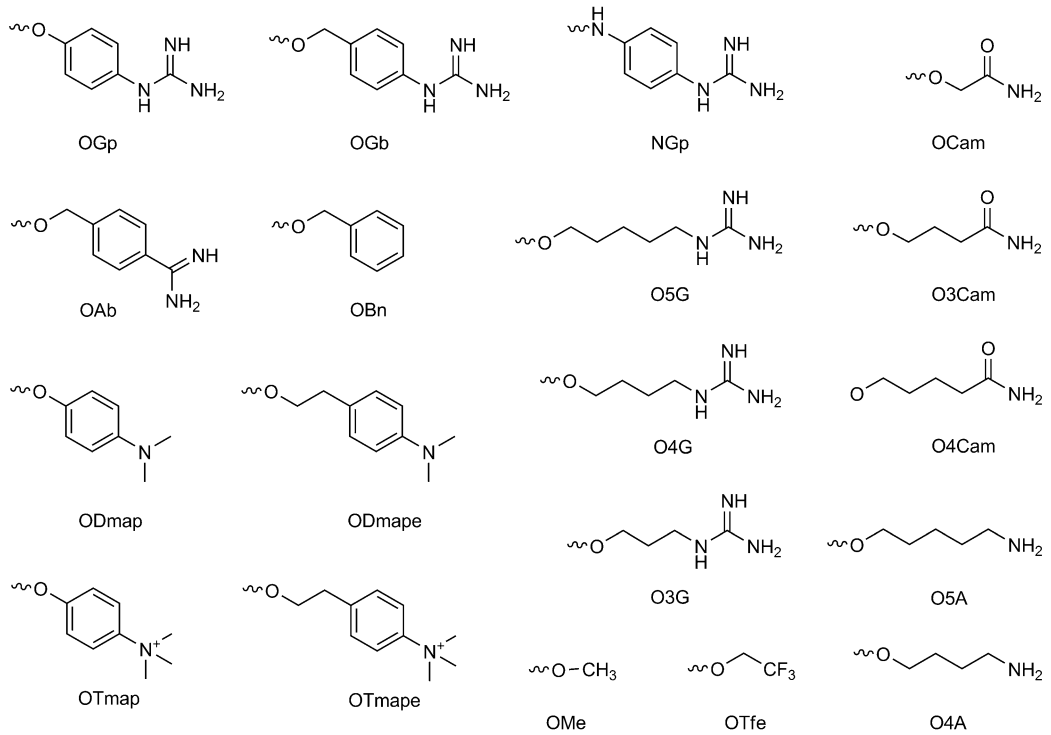
We recently reported that the OGp ester group can also be used in chemoenzymatic dipeptide synthesis under aqueous conditions with the protease papain.[12] In this case, the OGp moiety is likely not mimicking the natural substrate, because the guanidine group is predicted to bind to papain in a manner different from that of the arginine side chain. We named this phenomenon enzyme-specific activation, because no reaction takes place in the absence of the enzyme. From these findings we hypothesized that it might not be essential to have an arginine-like activating ester for papain, as long as sufficient specific interactions with the enzyme can be produced. A set of potentially enzyme-specific activating esters was therefore designed, synthesized, and evaluated for effectiveness in papain-catalyzed dipeptide formation.

Results and Discussion

Design of potentially enzyme-specific activating esters

In designing enzyme-specific activating esters for papain, several requirements had to be fulfilled. Firstly, sufficient functionalities (e.g., hydrogen bond donors or acceptors) were incorporated to allow for interactions with the binding groove of papain. This is also important for gaining insight into the spatial, electronic, and chemical requirements for papain-specific activating esters. Secondly, the stability to spontaneous ester hydrolysis needs to be improved. Chemical background hydrolysis for OGp esters is substantial (≈5% within 30 min)[12] and this is undesirable because it will increase when longer reaction times are required in more difficult couplings. Aliphatic or benzylic esters are considerably more stable than phenolic esters. Thirdly, the new activating esters must preferably be cheap and easy to synthesize. A chemoenzymatic route—using Alcalase-CLEA,[13] for example—would be particularly attractive in this respect. N-Protected amino acids can be converted into their alkyl or benzyl esters by this enzyme in near-anhydrous organic solvents. Furthermore, arylamides can be obtained by ester interconversion under the same conditions. The more labile phenyl esters are beyond the scope of this method.

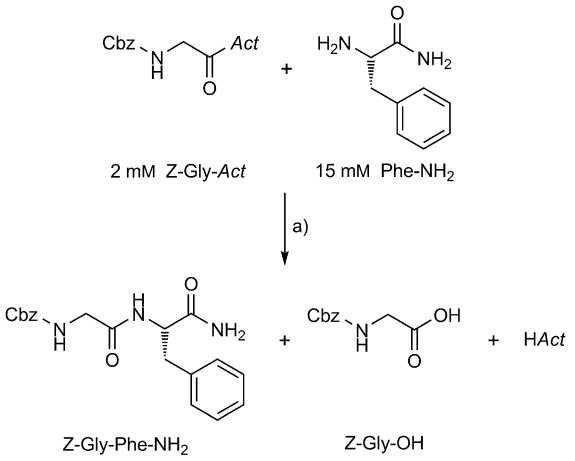
These considerations led to a set of potentially activating moieties, shown in Scheme 1. These were chemically synthesized as the corresponding Z-Gly-OH esters. In addition, four activated esters that have been described in the literature in combination with papain—OMe,[14] OCam,[14a,15] OTfe,[15] and OBn,[14a,16]—were also included for comparison. The synthesis details of all ester substrates can be found in the Supporting Information.



Scheme 1. Structures of potential activating esters. OGp: *p*-guanidinophenyl ester. OGb: *p*-guanidinobenzyl ester. NGp: *p*-guanidinobenzyl amide. OAb: *p*-amidinobenzyl ester. OBn: benzyl ester. OCam: carbamoylmethyl ester. O3Cam: carbamoylpropyl ester. O4Cam: carbamoylbutyl ester. O3G: 3-guanidinopropyl ester. O4G: 4-guanidinobutyl ester. O5G: 5-guanidinopentyl ester. ODmap: *p*-(dimethylamino)phenyl ester. OTmap: *p*-(trimethylammonium)phenyl ester. ODmape: *p*-(dimethylamino)phenethyl ester. OTmape: *p*-(trimethylammonium)phenethyl ester. O4A: 4-aminobutyl ester. O5A: 5-aminopentyl ester. OMe: methyl ester. OTfe: 2,2,2-trifluoroethyl ester.

Evaluation of potentially enzyme-specific activating esters

Next, the esters were evaluated for their ability to form the dipeptide Z-Gly-Phe-NH_2 with papain catalysis. The levels of conversion were determined by a previously described assay (Scheme 2).[12]



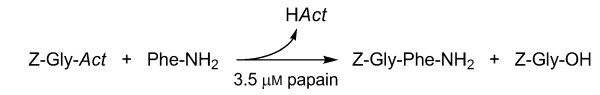
Scheme 2. Enzymatic dipeptide synthesis with papain. a) 3.5 μM papain, 2 mM *p*TSA, 10% (*v/v*) DMF, 0.2 M HEPES buffer (pH 8), 0.2 M NaCl, 20 mM $CaCl_2$.

Table 1 shows either the times it takes to reach 100% conversion or the levels of conversion after 3 h. Background hydrolysis of the esters was determined from blank reactions in which no papain was present. The indicated percentages of enzymatic synthesis and hydrolysis remained constant over time as measured after 24 h.

Ideally, the enzyme-specific activating group should give a fast reaction, a high percentage of synthesis, and no hydrolysis. In none of the entries are all these criteria met at the same time, except for the phenolic ODmap ester (entry 6). However, the liberated dimethylaminophenol suffers from decomposition, as deduced from the large amount of additional peaks in the HPLC chromatogram. The phenolic ester OTmap (entry 5) reacted comparably rapidly as OGp, but had a higher spontaneous hydrolysis rate. The introduction of two extra carbons (cf. ODmape and OTmape, entries 7 and 8, respectively) resulted in significant increases in reaction time. The benzyl ester (OBn, entry 17) was superior to OGp in many respects, except for the time needed to reach full conversion of the starting material. The other benzylic esters, OGb and OAb (entries 2 and 4, respectively), reacted even more slowly and showed different percentages of enzymatic hydrolysis, which is remarkable in view of their similarity. The introduction of an amide bond (entry 3) resulted in complete inactivity. Of the set of aliphatic potentially activating esters, only the activated OCam and OTfe esters (entries 14 and 18, respectively) reacted reasonably rapidly. As anticipated from their chemical properties, background hydrolysis was low for all aliphatic esters. The enzymatic hydrolysis levels, on the other hand, were much higher in all cases. These results are in line with earlier studies showing that the Z-Gly esters of OMe,[14b] OBn,[16] OCam,[14a] and OTfe[15] can be used in combination with papain. Because of the variation in documented reaction conditions—in organic solvent, in frozen aqueous solutions, varying equivalents, immobilized papain—it was, however, difficult to compare them properly. The fact that these reactions could not just be performed in buffer is consistent with the relatively high enzymatic hydrolysis values we find for these esters.

Although the enzymatic reactions proceed through the same acyl-enzyme intermediate (Z-Gly-papain), differences in S/H ratio are observed, This might be because the first step (acylation of the enzyme) is not the rate-determining step for each ester. Another explanation might be that the leaving groups have varying affinities for the enzyme and therefore influence the attack of the nucleophile (water or H-Phe-NH_2) to larger or smaller extents.

Table 1. Various experimentally tested Z-Gly-*Act* esters.[a]



		Experimental		Background	Enzymatic	
	Act	*t* [min]	Conv [%]	Z-Gly-OH [%]	Z-Gly-Phe-NH_2 [%]	Z-Gly-OH [%]
1	OGp	20	100	5.6	92.0	2.4
2	OGb	180	97	3.3	92.5	1.2
3	NGp	180	–	–	–	–
4	OAb	180	99	2.2	86.5	10.3
5	OTmap	15	100	8.4	91.6	0
6	ODmap	10	100	1.2	98.6	0.2
7	OTmape	180	61	0.4	58.1	2.5
8	ODmape	180	21	0.1	18.9	2.0
9	O3G	180	86	1.8	68.6	15.6
10	O4G	180	83	1.6	66.8	14.6
11	O5G	180	66	1.2	54.7	10.1
12	O4A	180	69	2.5	53.9	12.6
13	O5A	180	70	0.6	56.7	12.7
14	OCam	15	100	1.8	89.9	8.3
15	O3Cam	180	80	1.1	67.8	11.1
16	O4Cam	180	97	0.2	81.9	14.9
17	OBn	45	100	0	97.5	2.5
18	OTfe	15	100	1.8	91.5	6.7
19	OMe	180	70	1.5	58.8	9.7

[a] Conditions: Z-Gly-*Act* (2 mM), H-Phe-NH_2 (15 mM), papain (3.5 μM), HEPES buffer [pH 8.0, 0.2 M, NaCl (0.2 M), $CaCl_2$ (20 mM), DMF (10%, *v/v*)].

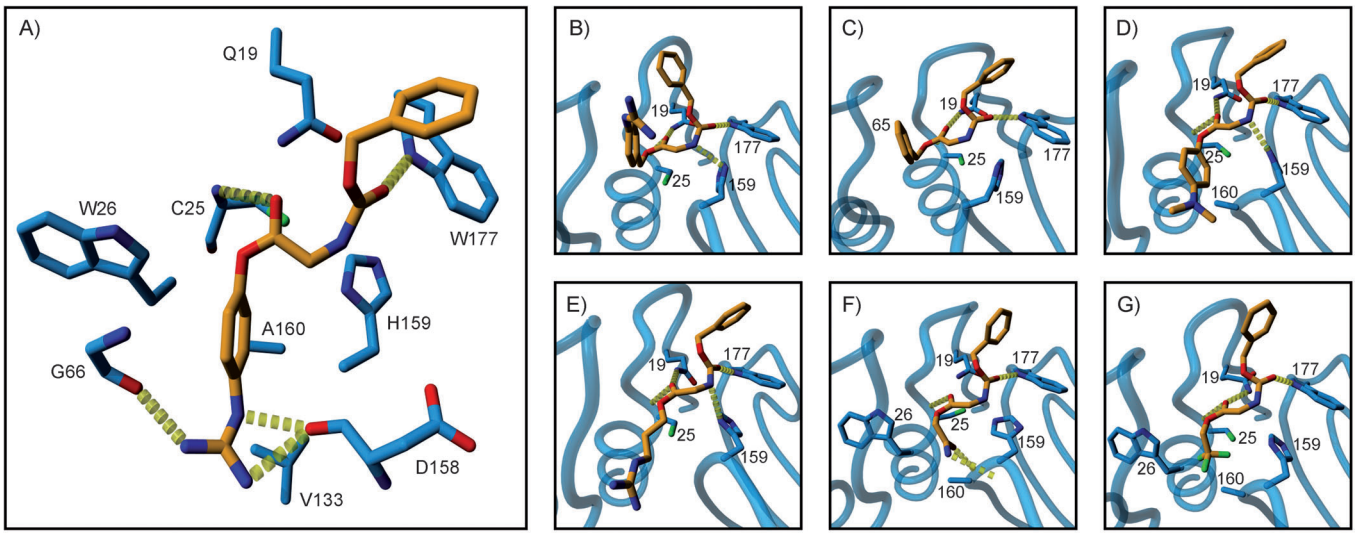


Figure 1. Molecular modeling of a selection of esters in papain. Hydrogen bonding interactions to functionally important amino acids in the papain active site are shown for A) Z-Gly-OGp, B) Z-Gly-OGb, C) Z-Gly-OBn, D) Z-Gly-ODmap, E) Z-Gly-O3G, F) Z-Gly-OCam, and G) Z-Gly-OTfe.

Docking of activating esters into the active site of papain

We recently developed a molecular model of the interactions of Z-X_{AA}-OGp esters with the active site of papain.[12] A similar molecular modeling study was performed for each of the remaining 18 substrates with the aid of the flexible docking program Fleksy.[17] Results were visualized and analyzed by use of the YASARA program. Several representative examples are shown in Figure 1 and discussed below.

Z-Gly-OGp (Figure 1 A) fits tightly in the papain active site, which is located in a groove between two domains.[12,18] The nucleophilic cysteine is as such positioned to attack the carbonyl carbon, whereas the carbonyl oxygen is situated in the oxyanion hole, which is made up of the side chain NH_2 group of Gln19 and the backbone amide of Cys25. The guanidinophenyl group is positioned in the groove, participating in several hydrogen bonding interactions.

In general, all the compounds discussed have the same N-to-C directionality as Z-Gly-OGp and fit the oxyanion hole.

The most noticeable difference between Z-Gly-OGp and Z-Gly-OGb (Figure 1 B) is that the guanidinobenzyl group is now pointing out of the groove as a consequence of the extra carbon atom. As a result, the hydrogen bonding interactions with the groove are lost, which might explain the significant change in activity. Similarly, in Z-Gly-OBn (Figure 1 C) the benzyl group is also oriented towards the solvent, but it can favorably interact with Gly65 at the edge of the groove. Z-Gly-ODmap (Figure 1 D), a phenolic ester, is nicely located in the groove and interacts with Tyr67 and Val133. Although the aliphatic Z-Gly-O3G (Figure 1 E) seems to be reasonably well situated in the groove, its reaction time is significantly longer than those of the phenolic esters. The small OCam moiety (Figure 1 F) participates in additional interactions with Trp26, Asp158, and Ala160 and fills the lower part of the peptide groove. High reactivity for OCam esters with α-chymotrypsin had already been reported to be attributable to orientation effects of hydrogen bonding interactions between the enzyme and the substrate.[19] Similarly, the OTfe group (Figure 1 G) exactly fills a hydrophobic cavity in the peptide groove, interacting with Trp26, Gly65. and Ala160.

These results imply that besides a good fit in the active site with sufficient interactions to the peptide groove and proper stabilization by the oxyanion hole, the leaving group ability of the activating ester is also of significant importance. Clearly, the relatively poor leaving group abilities of the aliphatic esters cannot be compensated for by the enzyme, whereas the differences between the much better phenolic leaving groups can be explained on the basis of their interactions with papain. The results also show that the good leaving groups OCam and OTfe are to some extent even further activated by the enzyme. The outcome with the benzyl ester is remarkable in our view, because the inferior leaving group ability is largely overcome by the enzyme-specific activation.

Scope of amino acid donor in Z-X_{AA}-OBn and Z-X_{AA}-ODmap

The high levels of conversion, combined with low hydrolysis rates, observed with the readily accessible ODmap and OBn esters led us to select them for further exploration. In particular, in order to probe the more general applicability of the concept of enzyme-specific activation, we investigated whether the relatively broad range of amino acid donors determined for OGp[12] would be maintained with ODmap and OBn. Z-X_{AA}-ODmap and Z-X_{AA}-OBn esters were synthesized in a procedure analogous to that followed for OGp esters, from a representative set of amino acids with appropriate acid-labile side chain protection and evaluated in the enzymatic assay. In the case of OBn esters, the amount of papain was doubled to compensate for the slower reaction rate that had been observed with Z-Gly-OBn. The identities of the products in the enzymatic reactions were confirmed by chemical synthesis of reference compounds and LC-MS analysis.

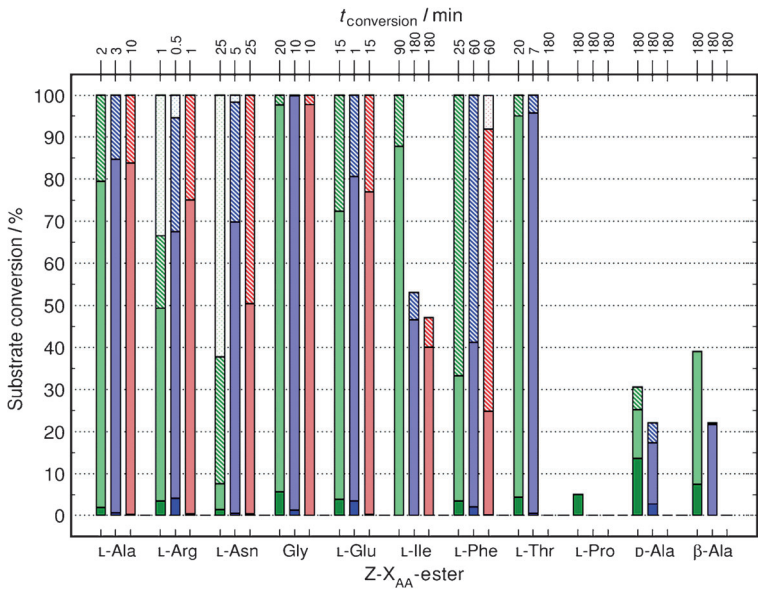


Figure 2. Product distributions for papain-catalyzed dipeptide synthesis for the three ester types OGp (green), ODmap (blue), and OBn (red). ■: spontaneous hydrolysis, ■: enzymatic synthesis, ▧: enzymatic hydrolysis, □: side product. Results are clustered per amino acid. Conditions: Z-X_{AA}-*Act* (2 mM), H-Phe-NH_2 (15 mM), papain (3.5 μM), HEPES buffer (pH 8.0, 0.2 M), NaCl (0.2 M), DMF (10%, *v/v*). For reactions with Z-X_{AA}-OBn double quantities of papain were used (7.0 μM). The dipeptide products Z-IF-NH_2 and Z-NF-NH_2 precipitated during the reaction, so the yields were estimated, Z-RF-NH_2 underwent secondary hydrolysis after 1 min to give Z-R-OH + H-F-NH_2, and Z-FF-NH_2 was enzymatically converted into Z-FF-OH in the OBn case.[20]

The distribution of background hydrolysis, enzymatic synthesis, enzymatic hydrolysis, and in some cases side product formation for each of the studied esters is shown in Figure 2 (the corresponding tables are included in the Supporting Information). The background hydrolysis was determined from a blank reaction with no papain present. The time indicated above each bar is either that required to reach 100% conversion, or that after which regular monitoring was stopped (3 h). The indicated product distribution remained constant over time, as measured after 24 h, unless stated otherwise.

For ease of comparison, the results for Z-X_{AA}-OGp[12] are also included in the diagram, and the data are clustered per amino acid. Some trends can be distinguished: generally, the percentages of enzymatic synthesis (■) over hydrolysis (▧) are comparable for each individual amino acid independent of the ester. This was to be expected because the binding pocket for the amino acid did not change. A remarkable exception in this respect is the complete inactivity of Z-L-Thr-OBn towards papain, which might be due to steric effects.

The spontaneous background hydrolysis (■) decreased on going from the OGp ester to the ODmap and OBn esters, which correlates well with the decreasing leaving group abilities of these esters. The nonenzymatic cyclic side product formation (□; a piperidone from Arg and a succinimide from Asn) dropped drastically in this order for the same reason. All of the OBn esters reacted considerably more slowly than the OGp and ODmap esters, despite the double quantities of papain. This difference in reactivity became even more apparent for the more challenging amino acids Z-L-Ile-OH, Z-D-Ala-OH, and Z-β-Ala-OH. Increasing the reaction time for the corresponding OBn esters did not result in significantly higher conversions, but dipeptide synthesis occurred when the quantities of papain were simultaneously increased by a further factor of ten (Table 2).

The scope of the amino acid donor is comparably broad for ODmap and OGp esters. The OBn ester group also enables the papain-catalyzed synthesis of various dipeptides, provided that the amount of enzyme is increased. This seems an acceptable trade-off given the fact that OBn esters are readily accessible, even with an enzymatic approach.

Table 2. Various Z-X_{AA}-OBn esters experimentally tested with a high papain concentration.[a]

	Amino acid	Experimental *t* [h]	Conv [%]	Background Z-X_{AA}-OH [%]	Enzymatic Z-X_{AA}-Phe-NH_2 [%]	Z-X_{AA}-OH [%]
1	L-Ile	6	100	–	30.9 (85.5)[b]	14.5
2	D-Ala	10	100	0.8	51.7	47.5
3	β-Ala	24	26	–	25.4	0.6

[a] Conditions: Z-X_{AA}-OBn (2 mM), H-Phe-NH_2 (15 mM), papain (70 μM), HEPES buffer (pH 8.0, 0.2 M), NaCl (0.2 M), $CaCl_2$ (20 mM), DMF (10%, *v/v*). [b] Dipeptide product precipitates during reaction; estimated yield is given in parentheses.

Synthesis of two selected dipeptides on preparative scale

The applicability of the enzyme-specific activation strategy was illustrated by means of preparative scale syntheses of two bioactive peptides: Z-Gly-Phe-NH_2, with Z-Gly-ODmap as activating ester, and H-Glu-Trp-OH starting from enzymatically synthesized Z-L-Glu-OBn. The first dipeptide is a substrate for metalloendoproteases and blocks the exocytotic release of histamine and catecholamines from mast cells and adrenal chromaffin cells.[21] Furthermore, it interferes with insulin processing and inhibits glucose transport in adipocytes.[22] The second is also known as thymogen, oglufanide, or IM862, a naturally occurring immunomodulator, which is a potent anti-angiogenic agent and normalizes the immune system function of immunocompromised individuals.[1a,23]

Firstly, the small-scale experiment with Z-Gly-ODmap and H-Phe-NH_2 (Table 3, entry 1) was repeated with 100 mg acyl donor instead of 0.25 mg, resulting in a longer reaction time and a slightly increased amount of hydrolysis as determined by HPLC analysis of a sample from the reaction mixture (entry 2). The desired dipeptide was isolated in 74% yield after column chromatography. Next, the excess of H-Phe-NH_2 was decreased to arrive at equimolar amounts of acyl donor and acceptor (entry 3). At this point, additional DMF was required to keep all compounds in solution, and the papain concentration was increased tenfold to compensate for the anticipated

loss of activity. These conditions resulted in a faster reaction, a somewhat better S/H ratio, and an isolated yield of 79%. Finally, this reaction was performed on a gram scale (entry 4). Despite the use of a ten times higher papain concentration, the reaction was not complete after 75 min, but the product was purified nevertheless. The isolated yield of 74% was equal to the yield calculated from the level of conversion indicated by HPLC.

The synthesis of the second dipeptide was envisioned as depicted in Scheme 3. The chemoenzymatic method developed by Nuijens was employed to benzylate Z-Glu-OH selectively at the α-position and the activating ester was obtained in 96% yield.[13a] This synthesis clearly emphasizes the advantage of OBn as an activating ester over phenolic esters, which cannot be enzymatically prepared. H-Trp-OBn was selected as the nucleophile to enable the simultaneous removal of both protecting groups, but with the risk of repeated coupling due to the continued presence of the OBn group, which is recognized by papain.

Test experiments on preparative scales indicated that multiple addition of H-Trp-OBn did indeed occur, but that it could be minimized by stopping the reaction in time. These experiments also showed that the reaction proceeds much more rapidly but with a worse S/H ratio than in the case of the previous dipeptide. This led us to conduct a 500 mg scale experiment with a 1:1 donor/acceptor ratio and a papain concentration of 8.8 μM. Under these conditions, HPLC analysis showed full conversion within 45 min and an S/H ratio of 66:27; additionally, a 7% yield of tripeptide was formed. The desired dipeptide was isolated in pure form in 60% yield after column chromatography. Subjection of the protected dipeptide to hydrogenolysis conditions provided biologically active H-Glu-Trp-OH in 87% yield.

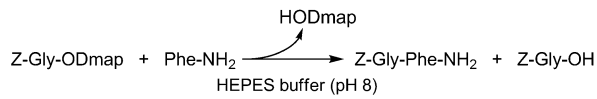
Conclusion

We have shown that papain-catalyzed dipeptide synthesis can also be performed successfully with activating esters that do not resemble arginine. Out of a set of potentially activating esters, both the ODmap and OBn esters appeared to be suitable replacements for OGp, with the added benefits of 1) being simpler in structure and therefore cheaper and easy to synthesize, and 2) giving smaller amounts of undesired hydrolysis products. The scopes of the ODmap and OBn acyl donors were compared with that of the OGp esters, and it was found that the synthesis to hydrolysis ratios are variable for the different amino acids, but rather were essentially independent of the ester involved. The reactions with OBn esters required increased quantities of papain to afford similar reaction rates. The applicability of the ODmap and OBn esters was validated in preparative-scale syntheses of two biologically active dipeptides.

The computational model of papain, which was originally built for OGp, was effectively applied in a docking study to provide insight into the differences in reactivity of all the potentially activating esters. This exercise showed that besides a proper fit in the active site, the leaving group ability is also important. To shed light on this intricate relationship, further studies, both computational and experimental, are currently underway. The balance between a good leaving-group ability to enable the enzymatic reaction, a stable ester that does not undergo rapid spontaneous hydrolysis, and the extent to which the ester is activated by the enzyme seems to be delicate.

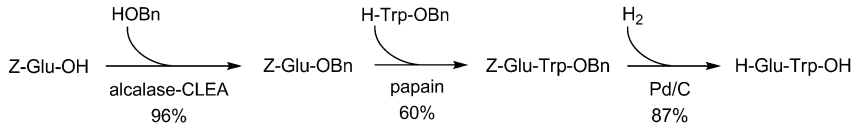
Experimental Section

Table 3. Reaction conditions and yields of preparative-scale syntheses of Z-Gly-Phe-NH_2.



	Scale [mg]	D/A[a] [mM]	DMF [%, *v/v*]	Papain [μM]	Time [min]	Conv.[b] [%]	S/H ratio[c] [%]	Yield[d] [%]
1	0.25	2:15	10	3.5	10	100	98:2	n.a.
2	100	2:15	10	3.5	120	97	85:12	74
3	100	15:15	20	35	60	99	90:9	79
4	1000	15:15	20	350	75	89	74:15	74

[a] Donor to acceptor ratio Z-Gly-ODmap/H-Phe-NH_2. [b] By HPLC. [c] Synthesis over hydrolysis ratio Z-Gly-Phe-NH_2/Z-Gly-OH. [d] Isolated yield of dipeptide after purification by flash column chromatography.



Scheme 3. Synthesis of bioactive dipeptide H-Glu-Trp-OH. Step 1: Z-Glu-OH, BnOH, Alcalase-CLEA, THF, molecular sieves (3 Å), 150 rpm, 37 °C. Step 2: Z-Glu-OBn (5 mM), H-Trp-OBn (5 mM), papain (8.8 μM), HEPES buffer (pH 8.0, 0.2 M), NaCl (0.2 M), $CaCl_2$ (20 mM), DMF (15%, *v/v*), 25 °C. Step 3: H-Cube, Pd/C (10%), H_2 (10 bar), flow rate 1 mL min^{-1}, RT.

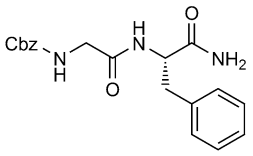
Synthesis: See the Supporting Information for a detailed description of the synthetic procedures for the activating esters and product characterization.

Enzymatic synthesis of dipeptides on preparative scale: The acyl donor, the acyl acceptor, and *p*TSA (as an internal standard) were placed in a round-bottomed flask and dissolved in a small amount of DMF, to which was added HEPES buffer (pH 8, with a final concentration of 0.2 M) containing NaCl (0.2 M) and $CaCl_2$ (20 mM). Finally, papain, activated with dithiothreitol (DTT) in phosphate buffer (0.1 M, pH 6.5) containing EDTA (2.5 mM), was added to start the reaction. The mixture was stirred at 25 °C until total conversion of the starting material was observed, which was determined by HPLC. The reaction mixture was then extracted with EtOAc (3×). The combined organic

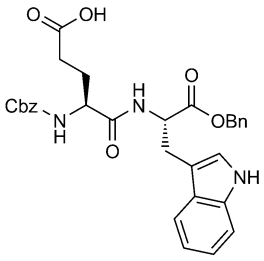
Table 4. Enzymatic synthesis of dipeptides on preparative scales.

Acyl donor	[mg]	Acyl acceptor	[mg]	*p*TSA [mg]	DMF [mL]	Buffer [mL]	Milli-Q [mL]	Papain [mg]
Z-Gly-ODmap	99	H-Phe-NH_2	443	57	15	75	60	12
Z-Gly-ODmap	99	H-Phe-NH_2	59	7.6	4	10	6	16
Z-Gly-ODmap	985	H-Phe-NH_2	59	76	40	100	60	160
Z-Glu-OBn	557	H-Trp-OBn	496	114	45	150	105	60

layers were washed with brine, dried over sodium sulfate, and concentrated to dryness. Table 4 summarizes the results.



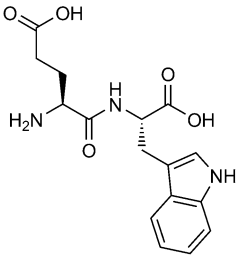
***N*$^{\alpha}$-Cbz-Glycine-phenylalanine amide (Z-Gly-Phe-NH_2)**: The product was obtained as a white solid after purification by column chromatography (MeOH in CH_2Cl_2, 2→5%). M.p. 143.0 °C; R_f = 0.45 (10% MeOH in CH_2Cl_2); $[\alpha]_D^{20}$ = +7.1 (*c* = 2.60, MeOH); ^{1}H NMR (CD_3CN, 400 MHz): δ = 7.42–7.18 (m, 10H), 6.88 (d, *J* = 7.7 Hz; NH), 6.44 (brs; NH), 5.98 (brt; NH), 5.83 (brs; NH), 5.07 (s, 2H), 4.52 (ddd, *J* = 5.2, 8.3, 8.3 Hz, 1H), 3.67 (dd, *J* = 6.1. 17.0 Hz, 1H), 3.62 (dd, *J* = 6.0. 16.9 Hz, 1H), 3.13 (dd, *J* = 5.1, 14.0 Hz, 1H), 2.89 ppm (dd, *J* = 8.5, 14.0 Hz, 1H); ^{13}C NMR (CD_3CN, 75 MHz): δ = 173.8, 170.1, 157.7, 138.4, 137.9, 130.2, 129.4, 129.2, 128.9, 128.7, 127.5, 67.3, 54.9, 44.9, 38.2 ppm; IR (film): $\tilde{\nu}$ = 3460, 3299, 3062, 3032, 1712, 1681, 1644, 1563, 1529, 1284, 1250, 1219, 1172, 1045, 992, 728, 697 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{19}H_{21}N_3NaO_4$: 378.1430 $[M+Na]^+$, found: 378.1443.



***N*$^{\alpha}$-Cbz-glutamate-tryptophan benzyl ester (Z-Glu-Trp-OBn)**: The product was obtained as a white solid after purification by column chromatography (AcOH/MeOH/EtOAc 1:5:94) and lyophilization from dioxane. M.p. = 48.7 °C; R_f = 0.50 (AcOH/MeOH/EtOAc 1:25:474); $[\alpha]_D^{20}$ = −13.8 (*c* = 1.00, MeOH); ^{1}H NMR (CD_3CN, 400 MHz): δ = 9.17 (s; NH), 7.51 (d, *J* = 7.9 Hz; NH), 7.41–7.19 (m, 11H), 7.15–7.10 (m, 1H), 7.08–7.01 (m, 3H), 5.99 (d, *J* = 7.4 Hz; NH), 5.09–4.99 (m, 4H), 4.75–4.69 (m, 1H), 4.13–4.05 (m, 1H), 3.29–3.15 (m, 2H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.82–1.71 ppm (m, 2H); ^{13}C NMR (CD_3CN, 75 MHz): δ = 174.7, 172.4, 172.3, 157.1, 137.3, 136.8, 129.4, 129.1, 129.0, 128.8, 128.6, 128.3, 124.7, 122.5, 120.0, 119.2, 118.2, 112.2, 110.3, 67.6, 67.2, 55.1, 54.2, 30.4, 28.2, 28.1 ppm; IR (film): $\tilde{\nu}$ = 3312, 3058, 3031, 2963, 2918, 1714, 1664, 1528, 1455, 1342, 1213, 743, 698 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{31}H_{31}N_3O_7$: 558.2240 $[M+H]^+$, found: 558.2240.

Chemoenzymatic synthesis of Z-Glu-OBn: Before use, Alcalase-CLEA (3 g, Type OM, CLEA-Technologies, 580 U g^{-1}) was suspended in *t*BuOH (100 mL) and crushed with a spatula. After filtration, the enzyme was resuspended in methyl *tert*-butyl ether (MTBE; 50 mL), followed by filtration. Large enzyme particles were removed with a sieve (0.5 mm pore size). Molecular sieves (Acros, 3 Å, 8 to 12 mesh) were activated (200 °C under vacuum overnight), crushed, and sieved (0.5 mm pore size) to remove large particles.

Alcalase-CLEA (509 mg) was added to a solution of Z-L-Glu-OH (977 mg, 3.48 mmol, 1 equiv) in THF (9 mL) and benzyl alcohol (1 mL, 9.66 mmol, 2.8 equiv). The mixture was shaken at 37 °C at 150 rpm for 16 h in the presence of molecular sieves (3 Å). After filtration, the enzyme was washed by resuspension in THF (3×20 mL) followed by filtration. The combined organic layers were concentrated in vacuo and the resulting oil was purified by column chromatography with aminomethyl resin (TFA in CH_2Cl_2, 0→2.5%). The solution was concentrated, TFA was removed by co-evaporation with toluene (2×20 mL) and $CHCl_3$ (2×20 mL), and the product was lyophilized from MeCN/H_2O (3:1) to give Z-Glu-OBn(1.24 g, 96%) as a white solid. Spectral data were consistent with those reported in the Supporting Information for the chemically synthesized compound.



Glutamate-tryptophan (H-Glu-Trp-OH): Z-Glu-Trp-OBn (121 mg, 0.22 mmol, 1 equiv) was dissolved in H_2O in MeOH (20%, 12 mL) and hydrogenated in the presence of Pd/C (10%) in the H-Cube (ThalesNano) at room temperature, 1 mL min^{-1}, and 10 bar H_2 pressure. The product was obtained as a slightly pink solid after evaporation of the solvent in vacuo (63 mg, 87%). M.p. = 159.2 °C; R_f = 0.27 ($CHCl_3$/MeOH/NH_4OH 65:45:20); $[\alpha]_D^{20}$ = +22.4 (*c* = 0.31, DMSO); ^{1}H NMR (CD_3OD/D_2O, 400 MHz): δ = 7.64 (d, *J* = 7.8 Hz, 1H), 7.37 (d, *J* = 8.1 Hz, 1H), 7.18 (s, 1H), 7.11 (ddd, *J* = 1.1, 7.1, 8.1 Hz, 1H), 7.04 (ddd, *J* = 1.1, 7.1, 8.0 Hz, 1H), 4.61 (dd, *J* = 4.8, 8.7 Hz, 1H), 3.82 (dd, *J* = 5.2, 7.4 Hz, 1H), 3.39 (dd, *J* = 4.8, 14.7 Hz, 1H), 3.18 (dd, *J* = 8.8, 14.7 Hz, 1H), 2.52–2.36 (m, 2H), 2.16–1.95 ppm (m, 2H); ^{13}C NMR (CD_3OD/D_2O, 75 MHz): δ = 179.5, 177.5, 169.8, 137.7, 128.7, 124.7, 122.4, 119.8, 119.4, 112.4, 111.5, 56.9, 54.3, 33.3, 28.6, 28.4 ppm; IR (film): $\tilde{\nu}$ = 3213, 3067, 1693, 1665, 1597, 1548, 1483, 1455, 1414, 1339, 740 cm^{-1}; HRMS (ESI) *m/z* calcd for $C_{16}H_{20}N_3O_5$: 334.1403 $[M+H]^+$; found: 334.1391.

Computational studies: a detailed description of the computational analyses can be found in a previous article on this subject.[12]

Acknowledgements

This research was performed under the ACTS-NWO Integration of Biosynthesis & Organic Synthesis (IBOS) program. DSM Innovative Synthesis B.V. (Geleen, the Netherlands) is gratefully acknowledged for financial support. S.B.N. is supported by the Netherlands Organization for Scientific Research (NWO) through a VENI grant (700.58.410). Furthermore, Timo Nuijens (DSM) and Martin Broxterman are gratefully acknowledged for their help with the enzymatic synthesis of Z-Glu-OBn.

Keywords: enzymatic peptide synthesis · enzyme-specific activation · molecular modeling · papain · peptides

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Received: January 8, 2012
Published online on May 21, 2012

