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Quantitative enzymatic protection of D-amino acid methyl esters by exploiting 'relaxed' enantioselectivity of penicillin-G amidase in organic solvent

Chiara Carboni,[a,b] Peter J. L. M. Quaedflieg,[b] Quirinus B. Broxterman,[b] Paolo Linda[a] and Lucia Gardossi[a,*]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Trieste, Piazzale Europa 1, 34127 Trieste, Italy*
[b]*DSM Research, Life Sciences—Advanced Synthesis, Catalysis and Development, PO Box 18, 6160 MD Geleen, The Netherlands*

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Abstract—The lower enantioselectivity displayed by penicillin-G amidase (PGA) from *E. coli* in organic solvent has been exploited for developing a facile, fast and quantitative method for protection of esters of various D-amino acids via *N*-acylation. The feasibility of the deprotection of the acylated products was also demonstrated by employing PGA from two different sources in aqueous media. Experimental results are in agreement with previous calculations based on *in silico* models of the enzyme active site.

The application of biocatalysts in peptide chemistry offers several important advantages since stereo-, regio- and chemo-selective reactions take place under very mild conditions, so that the use of toxic and expensive chemicals for activation and (de-)protection are correspondingly reduced as well as operational hazards.[1,2] The methodology has not been fully exploited for possible synthesis of a number of biologically important peptides containing D-amino acids or other nonproteogenic amino acid derivatives. Antibiotic peptides, synthetic peptides of enhanced hormonal or neuronal activity and many prodrugs used in chemotherapy often contain D-amino acid residues.[3] Recombinant DNA technology is generally limited to the production of peptides containing only L-amino acids as, in general, they have been approached by protease catalysis.[1]

Protection and deprotection strategies are one of the crucial factors in determining the successful accomplishment of any peptide synthesis. Penicillin-G amidase (PGA) has become one of the enzymes of choice for protection/deprotection of amino groups[2,4,5] due to its high selectivity towards the phenylacetic group enabling the deprotection of the amino function in aqueous solution at ambient temperature without affecting other bonds or reactive groups.

The 3-D structure of PGA is well known and crystallographic data are accessible[6,7] which indicate how the enzyme presents a highly selective acylic sub-site for the phenylacetic group and an aminic sub-site characterised by a quite wide tolerance for substrates. The aminic sub-site, however, is responsible for enantio-discrimination,[8] so that the enzyme has been largely applied also in resolution of aliphatic alcohols,[9] amines[10,11] and especially amino acid derivatives.[12] In the case of amino acids and their derivatives, the L-enantiomer is the favourite substrate, both in acylation reaction[13] and in the hydrolysis of the corresponding *N*-acylated compounds.[14]

For instance, an enantioselectivity ratio (*E* value) of 280 was reported for the PGA mediated hydrolysis of *N*-phenylacetyl-AlaOMe and an *E* value of >1000 for *N*-phenylacetyl-PhGly.[14] Furthermore, it was demonstrated that D-TyrOEt is acylated only with unacceptably slow rate (7% after 44 h) even in the presence of an organic co-solvent.[15] Due to this strict L-enantioselectivity of PGA in aqueous media the N-protection of D-amino acids derivatives by PGA has never been disclosed.

Here we report, for the first time, on the PGA catalysed acylation of a series of D-amino acids esters in organic

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* Corresponding author. Tel.: +39 040558310; fax: +39 04052572; e-mail: gardossi@units.it

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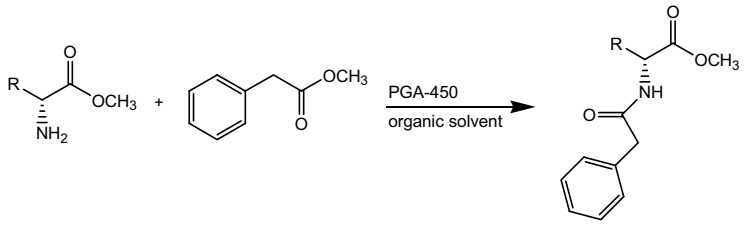

Scheme 1. *N*-acylation of D-amino acids esters catalysed by penicillin-G amidase.

solvent. The ability of PGA to accept the unfavoured D-enantiomer is related to a modification of the enantioselectivity of PGA in the presence of nonaqueous media. The use of an organic medium in enzymatically catalysed acylations not only causes a 'relaxation' of enzyme enantioselectivity but also prevents undesirable hydrolytic side reactions, so that equimolar amounts of acylating agents can be used. As a matter of fact, acylation in aqueous media requires the use of an excess of one of the reactants or extraction or precipitation of the product[11,12,15] to reverse the equilibrium towards synthesis. Although the presence of water is essential for preserving the activity of PGA, we have earlier demonstrated that PGA is active in various organic solvents as long as the water activity (a_w) of the reaction system is maintained above 0.4[4,16,17](Scheme 1).

In our previous study[18] concerning PGA mediated acylations in toluene we demonstrated that PGA is sufficiently L-enantioselective in this medium to resolve racemic PhGlyOMe with ee > 98%. However, kinetic measurements carried out on the separate enantiomers showed that the acylation rate for the D-enantiomer was not negligible, being 0.25 μmol h^{-1} U^{-1} as compared to 4.58 μmol h^{-1} U^{-1} determined for the L-enantiomer. More evidently, in another study concerning PGA enantioselectivity in toluene we reported an ee as low as 24% in the resolution of D,L-AlaOMe.[8]

These results, when compared to the data obtained by others in aqueous or partly aqueous media, prompted us to investigate the generality of this 'relaxed' L-enantioselectivity of PGA in organic solvent. Several studies reported the variation or even inversion of enzyme selectivity upon changing the reaction medium. According to most authors the influence of the reaction medium on enzyme selectivity can be ascribed to three different factors: (i) the alteration of the enzyme–substrate recognition process due to a variation in the enzyme conformation; (ii) the binding of solvent molecules inside the active site and (iii) altered energetics of substrate solvation.[19]

We first investigated whether D-amino acids methyl esters[20] could be smoothly and quantitatively acylated using immobilised PGA (PGA-450) and methyl phenylacetate as the acyl donor.

To this end a structurally versatile series (Table 1) of aliphatic and aromatic D-amino acids methyl esters (0.1 M solution in dry organic solvent) were reacted with 1.0 equiv of methyl phenylacetate in the presence of immobilised PGA-450 (2 g/mmol substrate); the activity of the enzyme was assured by controlling the water activity (a_w = 0.73–0.77) of the medium according to methods previously described.[17] As evident from Table 1, both aliphatic and aromatic substrates were quantitatively converted in a fairly short time frame.[21,22]

The complete conversion and the absence of side products allowed for the immediate isolation of the pure *N*-acylated D-amino acids methyl esters (in isolated yields ranging from 75% to 95%) upon removing of the enzyme by filtration and evaporation of the solvent. At the values of water activity employed for the enzymatic acylation (a_w = 0.73–0.77) no hydrolytic reaction was observed.

It must be noted that previous studies in aqueous media reported no reactivity of PGA towards D-Val.[13] Furthermore, in the specific case of the unnatural amino acid PhGly, of which the L-enantiomer is accepted by PGA both as nucleophile and as acyl donor, it was previously demonstrated that the D-enantiomer was accepted as acyl donor in aqueous media but not at all as nucleophile.[23] This is also the reason why during the PGA mediated synthesis of the antibiotics Cephalexin and Ampicillin, wherein D-PhGly esters or amides are used as acylating agents, the corresponding D,D-dipeptides are not formed whatsoever.

Table 1. Protection of various D-amino acids methyl esters catalysed by (immobilised) PGA-450 in toluene or dichloromethane at a_w = 0.73–0.77 using methyl phenylacetate as acyl donor

Entry[a]	Substrate	Solvent	T (°C)	Time for complete conversion (h)
1	D-SerOMe	Dichloromethane	30	24
2	D-ValOMe	Toluene	30	24
3	D-AlaOMe	Toluene	30	24
4	D-LeuOMe	Toluene	30	48
5	D-TyrOMe	Toluene	40	24
6	D-PheOMe	Toluene	30	72
7	D-PhGlyOMe	Toluene	30	72
8	D-PheOMe	Toluene	50	24[b]

[a] Reaction conditions: 1 mL of organic solvent, 200 mg PGA-450, 100 μmol of D-amino acids methyl ester, 100 μmol of methyl phenylacetate.

[b] Another portion of fresh enzyme (200 mg) was added after 6 h.

The results in Table 1 also clearly indicate that the acylation of the aromatic D-amino acids methyl esters is slower. This is in agreement with the structural basis of PGA enantio-discrimination which was reported in our previous study.[8] Computational investigation of the PGA active site demonstrated that in the case of the bulkier aromatic amino acids the stabilising effect of H-bonds between the acyl group and the PGA aminic sub-site effectively occurs only with the L-enantiomer, thus inducing enantio-discrimination. This is not the case with the smaller aliphatic amino acids, where both enantiomers can be accommodated inside the aminic sub-site so to acquire a conformation able to engage stabilising H-bonds with the active site. Thus, these theoretical calculations excellently explain the much lower reactivity of the aromatic D-amino acids compared to the aliphatic ones.

The difference in activities cannot be ascribed simply to solubility differences of the substrates since, for instance, D-SerOMe, which is the most polar and least soluble among the substrates considered, is completely acylated in 24 h even in dichloromethane, which had been demonstrated to be a less efficient solvent than toluene.[17] Finally, early studies on PGA had already demonstrated that the lower reactivity of the D-enantiomer was not ascribable to an inhibitory effect on PGA but only to kinetic factors.[15]

It should be noted that in the case of D-TyrOMe and D-PheOMe the higher enantioselectivity of PGA for the L-enantiomers can be partially overcome by speeding up the enzymatic acylation through a temperature increase to 40 or 50 °C, respectively.

Secondly, we investigated whether *N*-phenylacetyl-D-amino acids methyl esters could also be smoothly and quantitatively deprotected in aqueous solution using immobilised PGA from both *E. coli* (PGA-450) and *A. faecalis*. This latter amidase was selected because of the very high specific activity of its immobilised form (1390 U/g compared to 155 U/g of PGA-450) in order to increase the very low reaction rates which were expected for the hydrolytic reactions in aqueous medium. Furthermore, recent computational studies pointed out some important structural differences at the level of the aminic sub-site which induce to expect a wider substrate tolerance.[24] For the substrates tested (*N*-phenylacetyl-D-AlaOMe and *N*-phenylacetyl-D-PheOMe) the deprotection in buffered aqueous solution was complete within 24 h and 48 h, respectively, demonstrating the practical potential of the *N*-phenylacetyl protecting group for D-amino acids, for instance in peptide synthesis.[25]

In conclusion, a fast, efficient and versatile method was developed for the protection of D-amino acids esters by exploiting the lower enantioselectivity displayed by PGA in organic media. The procedure is of practical importance due to the absence of hydrolytic side products and the easy work-up. The results obtained are in agreement with previously reported theoretical calculations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2004.10.153. Materials for chemical synthesis; methods for enzyme dehydration as also reported in Ref. 17; procedure for the determination of enzyme activity as also described in Ref. 17; procedure for the determination of the water activity as also described in Ref. 17; chemical synthesis of the D-amino acids methyl esters starting from the corresponding D-amino acids, as also described in Ref. 20; preparation of the free amines starting from the corresponding hydrochloride salts.

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21. *Materials and methods*: PGA-450 was a generous gift of Boehringer–Mannheim. It consists of penicillin-G amidase from *E. coli* covalently immobilised on a polymer the chemical nature of which is not disclosed by the producer. The PGA-450 used in the present study had a water content of 62 wt% (determined by Karl–Fischer titration) and an activity, before partial dehydration, of 155 U/g corresponding to 408 U/g_{dry} (please refer to supplementary data and Ref. 17). For the *N*-acylation reactions, the PGA-450 was partially dehydrated before use with the aid of Celite R-640® as previously described.[17] The PGA from *A. faecalis*, covalently immobilised on a polymer support the chemical nature of which is not disclosed, was from DSM Anti-Infectives (Delft, The Netherlands). The enzymatic activity was 1390 U/g. ^{1}H NMR spectra were recorded at 200 MHz using a Varian–Gemini 200 spectrometer.
22. *N-acylation of* D-*amino acids methyl esters using PGA-450*: PGA-450 (200 mg) was suspended in toluene or CH_2Cl_2 (1 mL) and the system was equilibrated for 24 h (a_w = 0.73–0.77) at 30 °C. Subsequently, the D-amino acids methyl ester (100 μmol of free base) and methyl phenylacetate (100 μmol) were added. The course of the reactions was monitored with TLC (eluent: EtOAc/*n*-hexane 7:3 (v/v)). After full conversion of the D-amino acids methyl ester and methyl phenylacetate to the *N*-phenylacetyl-D-amino acids methyl esters (see Table 1), the enzyme was filtered off and the organic solvent evaporated in vacuo. ^{1}H NMR (DMSO-d_6), δ (ppm): PhAc-D-ValOMe: 0.9 (6H, m, –CH(CH_3)$_2$), 2.0 (1H, m, –CH(CH_3)$_2$), 3.5 (2H, d, –CH_2Ph), 3.6 (3H, s, –OCH_3), 4.2 (1H, dd, –CHNH), 7.35 (5H, m, –Ph), 8.4 (1H, d, –NHCO). PhAc-D-AlaOMe: 1.3 (3H, d, –CH_3), 3.4 (2H, d, –CH_2Ph), 3.6 (3H, s, –OCH_3), 4.3 (1H, dd, –CHNH), 7.25 (5H, m, –Ph), 8.55 (1H, d, –NHCO). PhAc-D-PheOMe: 3.0 (2H, m, –CH_2Ph), 3.4 (2H, d, –CH_2Ph), 3.6 (3H, s, –OCH_3), 4.5 (1H, m, –CHNH), 7–7.4 (10H, m, 2 × Ph), 8.6 (1H, d, –NHCO). PhAc-D-PhGlyOMe: 3.55 (2H, s, –CH_2Ph), 3.6 (3H, s, –OCH_3), 3.7 (1H, d, –CH–), 7–7.5 (10H, m, 2 × Ph), 9.0 (1H, d, –NH–). PhAc-D-LeuOMe: 0.9 (6H, m, –CH(CH_3)$_2$, 1.3 (1H, m, –CH(CH_3)$_2$), 1.5 (2H, m, CH_2CH(CH_3)$_2$), 3.45 (2H, d, –CH_2Ph), 4.2 (1H, m, –CHNH), 7.35 (5H, m, –Ph), 8.5 (1H, d, –NHCO). PhAc-D-SerOMe: 3.25 (2H, m, –CH_2OH), 3.5 (2H, d, –CH_2Ph), 3.65 (3H, s, –OCH_3), 4.4 (1H, dd, –CHNH), 7.35 (5H, m, –Ph), 8.4 (1H, d, –NHCO). PhAc-D-ThrOMe: 1.1 (3H, d, –CH_3), 3.6 (3H, s, –OCH_3), 3.8 (2H, s, –CH_2Ph), 4.3 (1H, t, –CH), 4.5 (1H, m, –CHOH) 7–7.5 (5 H, m, Ph), 8.0 (1H, s, –OH), 8.5 (1H, d, –NH–). PhAc-D-TyrOMe: 2.78 (2H, m, –CH_2Ph), 3.45 (2H, d, –CH_2Ph), 3.6 (3H, s, –OCH_3), 4.4 (1H, m, –CHNH), 6.45–7.4 (9H, m, 2 × Ph), 8.45 (1H, d, –NHCO), 9.2 (1H, s, –OH).
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25. *PGA catalysed hydrolysis of PhAc-*D-*AlaOMe and PhAc-*D-*PheOMe*: 200 mg of PGA-450 (from *E. coli*) or 200 mg of PGA from *A. faecalis* were added to a suspension of the *N*-acylated D-amino acids methyl ester (100 mg) in 5 mL aq sodium phosphate buffer (50 mM, pH = 8.0) at 30 °C. The reactions were monitored by TLC (eluent: 1-butanol/acetic acid/ethyl acetate/water 1:1:1:1 (v/v/v/v)). For PhAc-D-AlaOMe the deprotection reaction was complete after 24 h using both enzymes (product detectable already after 10 min with PGA from *A. faecalis* and after 1 h with PGA from *E. coli*). After 24 h, the reaction mixtures were filtrated and the filtrates evaporated in vacuo and analysed by NMR confirming the complete deprotection into D-AlaOMe. In the case of PhAc-D-PheOMe the solubility in water was considerably lower, so that an emulsion was obtained and the deprotection reaction took longer time. After 24 h both reactions were not complete and a new portion of the enzymes (200 mg) was added and the temperature increased to 40 °C. After 24 h of further stirring the deprotections were complete as was also confirmed by NMR.