Tetrahedron Letters 52 (2011) 5398–5402

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Alcaligenes faecalis penicillin G acylase-catalyzed enantioselective acylation of DL-phenylalanine and derivatives in aqueous medium

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ARTICLE INFO

Article history:
Received 8 April 2011
Revised 31 July 2011
Accepted 9 August 2011
Available online 16 August 2011

Keywords:
Penicillin G acylase
Acylation
DL-Phenylalanine
Aqueous medium

ABSTRACT

A new strategy based on enantioselective acylation properties of relatively unknown penicillin G acylase from *Alcaligenes faecalis* has been developed for the production of pharmacologically interesting enantiomerically pure D-phenylalanine. In order to get high reaction rate and enantioselectivity, two key factors (pH and temperature) and eight different acyl donors were optimized, and the optimal acylation reaction was carried out at pH 10, 35 °C, using phenylacetamide as the acyl donor. This enantioselective acylating method is also illustrated by the effective production of five different *p*-substituted phenylalanine derivatives in enantiopure.

Enantiopure D-phenylalanine and its *p*-substituted derivatives (Fig. 1) are important chiral building blocks for the preparation of some pharmaceuticals. D-phenylalanine is an intermediate in the synthesis of antiviral Fosamprenavir[1] and Saquinavir,[2] antineoplastic Clientide,[3] antidiabetic Nateglinide[4] and anticoagulant Melagatran.[5] D-Tyrosine [4-hydroxy-D-phenylalanine] is a constituent of the Tractocile, a potent abortion prevention agent.[6] 4-Methyl-D-phenylalanine is used in the synthesis of enkephalinol analogs.[7] In addition, 4-fluoro-D-phenylalanine, 4-chloro-D-phenylalanine and 4-nitro-D-phenylalanine are precursors of antiemetic Aprepitant, antineoplastic Abarelix and analgesic Zolmitriptan, respectively.[8–10]

In this regard, the development of methods to access enantiopure D-phenylalanine and its *p*-substituted derivatives is of great interest. Methods combined with biocatalytic technologies have been described for this purpose. For instance, Isabel group and Takashi group had, respectively, reported synthesizing methods in biocatalytic ways, which were from the corresponding DL-amino acid amides with L-amidase and from DL-amino acid esters using protease.[11,12] However, additional chemical hydrolysis steps and necessity of preparing DL-amino acid amides or esters before enzymatic hydrolysis limit these processes in industrial application.

Penicillin G acylase (PGA) has traditionally been used as an industrial catalyst for the production of semi-synthetic antibiotics.[13] Moreover, PGA is also used in the synthesis of enantiopure L-amino acids through enantioselective enzymatic hydrolysis of *N*-phenylacetyl-DL-amino acids in aqueous medium.[14] Nevertheless, additional chemical phenylacetyl-protection and deprotection steps are required if an D-amino acid is the desired product, which make PGA mediated hydrolysis method tedious and inefficient (Scheme 1). Cole showed that PGA can also be applied in the synthetic direction and thus provided a facile route of D-amino acids production based on enzymatic acylation of α-amino functionality using phenylacetic acid analogs as acylating agents.[15] PGA catalyzed acylation reaction is always competed by hydrolytic reaction in aqueous medium. The hydrolysis of acylating agents and produced *N*-phenylacetyl-L-amino acids brings the difficulty to drive acylation to completion and thereby leads to relatively low enantiomeric excess value of the remaining substrates (D-amino acids).[16] Many strategies have been proposed to favor acylation over hydrolysis. Using organic solvents as the reaction medium is the most often and thoroughly studied method.[17–20] However, massive amount of enzyme is required to perform the acylation and the reaction time tends to be long because of the low activity of PGA in organic solvents. Besides, amino acids must be first

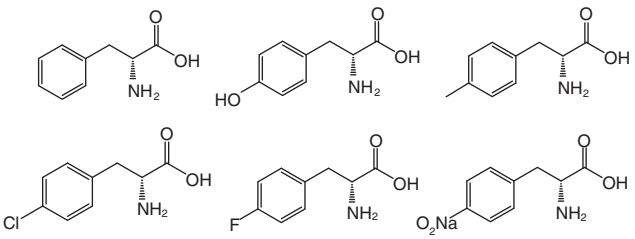

Figure 1. D-Phenylalanine and some *p*-substituted derivatives.

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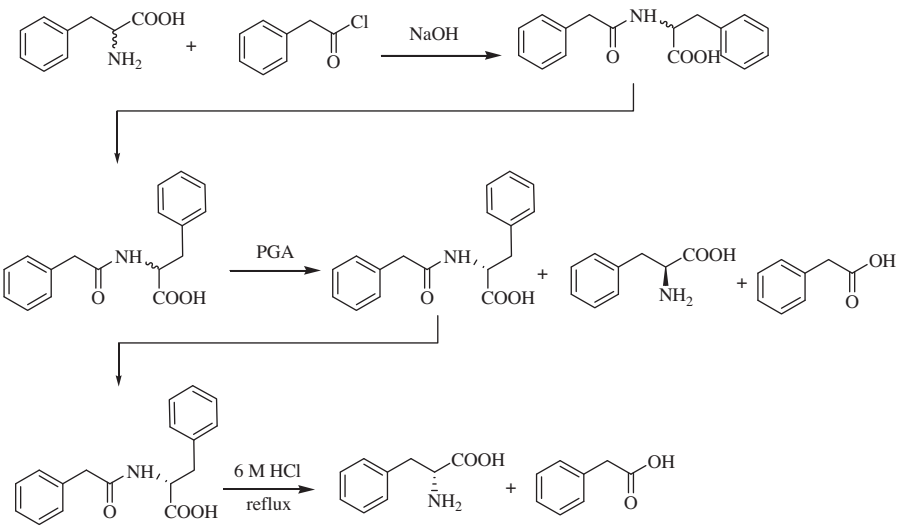
doi:10.1016/j.tetlet.2011.08.056



Scheme 1. Production of enantiomerically pure D-phenylalanine by PGA hydrolysis.

transformed into esters to render them soluble in organic solvents. So the use of aqueous medium is preferred option from the standpoint of green chemistry. We had successfully developed an approach for the production of D-β-phenylalanine in aqueous medium based on enantioselective acylation property of PGA from Escherichia coli, which proved that high enantiomeric excess value of substrate and improved synthesis/hydrolysis ratio (*s*/*h* ratio) could be achieved through optimization of acylation conditions involving temperature and pH.[21] Besides, we also found alkaline medium like pH 10 was more suitable for acylation reaction catalyzed by PGA since ionization of α-amino functionality of β-phenylalanine was reduced which made it a more efficient nucleophile than water towards acyl-enzyme intermediate. And thus synthetic efficiency was improved. So a PGA which is stable and active in alkality is favorable. In respect of this, our attention was drawn towards *Alcaligenes faecalis* PGA.

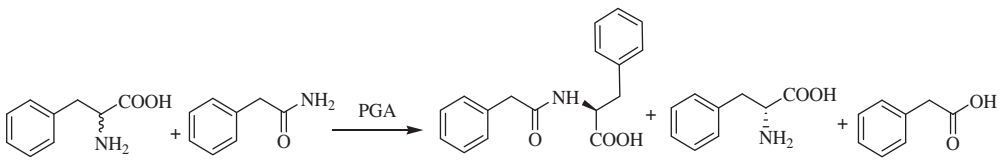
A. faecalis PGA is structurally similar to the well studied PGA from *E. coli*.[22] Compared with *E. coli* PGA, however, *A. faecalis* PGA bears a broader pH optimum of 8–10 and maintains nearly 80% of its maximum catalytic activity at pH 11.[23] Besides, *A. faecalis* PGA displays higher thermostability (due to the presence of a disulfide), higher catalytic activity and *s*/*h* ratio.[24] All of these show that the *A. faecalis* PGA may be an ideal biocatalyst for amino acids acylation reaction. Dorel and co-workers have investigated the *A. faecalis* PGA catalyzed enantioselective acylation of amines in aqueous medium.[23] To our knowledge, nevertheless, enantioselective acylation of amino acids catalyzed by *A. faecalis* PGA in aqueous medium has not been reported. Herein, we report a simple methodology for obtaining enantiomerically pure D-phenylalanine and derivatives, which is based on the principle of enantioselective acylation of racemic amino acids with *A. faecalis* PGA immobilized on oxirane acrylic beads (diam. 150–200 μm) in aqueous medium (Scheme 2). In this work, the main influence parameters were optimized and the acylating agents were screened.

The reaction conditions were optimized using DL-phenylalanine (**1a**) as substrate and phenylacetamide (**2a**) as acyl donor. Our studies were focused on the effects of pH and temperature on the acylation of DL-phenylalanine, and the results are summarized in Table 1. In all entries ee_p were above 99% and not affected by pH or temperature, while ee_s was relatively lower. This is mainly because of the hydrolysis of phenylacetamide and produced *N*-phenylacetyl-L-phenylalanine.[16] The maximum ee_s (92%) and substrate conversion (48%) were attained at pH 10 and 35 °C. Relatively higher pH and temperature could lead to higher conversion of substrate and ee_s except for entries 4 and 9 because the stability of immobilized PGA can be affected by harsh conditions (pH 11 and 40 °C). The results could be explained to a large extent on the basis of diffusion limitation mechanism. As we known, in immobilized preparations, diffusion limitation in the carrier tends to increase hydrolysis due to depletion of the reactants and accumulation of the products in the carrier beads.[25] In this work, *A. faecalis* PGA was immobilized on porous oxirane acrylic beads (diam. 150–200 μm). Most of *A. faecalis* PGA molecules were immobilized inside the porous structure of the carrier beads. Reactants (phenyl-

Table 1
Effect of pH and temperature on the *A. faecalis* PGA catalyzed acylation of DL-phenylalanine

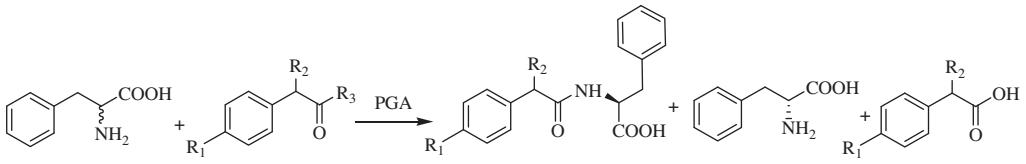
Entry	pH	Temperature (°C)	t^a (min)	$ee_p{}^b$ (%)	$ee_s{}^b$ (%)	c^c (%)
1	8	30	280	>99	40	29
2	9	30	240	>99	74	43
3	10	30	200	>99	86	46
4	11	30	300	>99	77	43
5	10	20	240	>99	83	45
6	10	25	240	>99	84	46
7	10	30	200	>99	86	46
8	10	35	220	>99	92	48
9	10	40	300	>99	91	48

[a] Reaction time at maximum DL-phenylalanine conversion.
[b] Determined by chiral HPLC.
[c] Deduced from the ee of the substrate (ee_s) and the product (ee_p): $c = ee_s/(ee_s + ee_p)$.

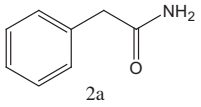
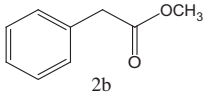
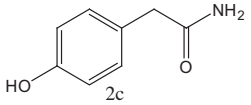
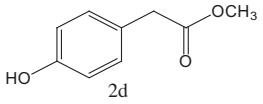
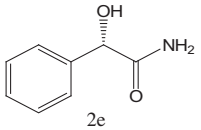
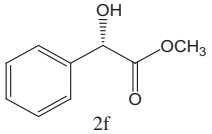
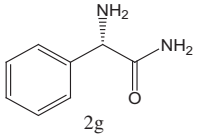
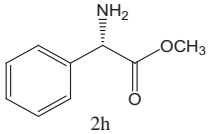


Scheme 2. Production of enantiomerically pure D-phenylalanine by PGA acylation.

Table 2
Effect of different acyl donors on the *A. faecalis* PGA catalyzed acylation of DL-phenylalanine



1a 2a-2h D-1a

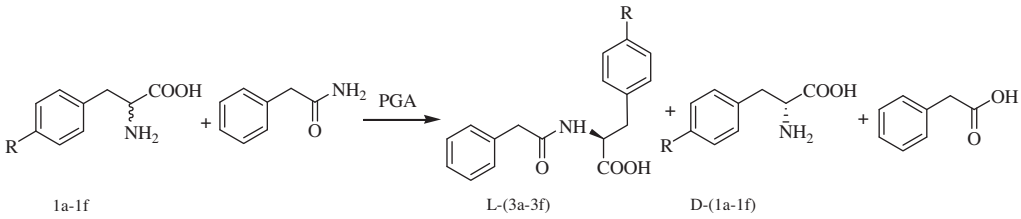
Entry	Acyl donor	equiv	t[a] (min)	ee_p[b](%)	ee_s[b](%)	c[c] (%)	$(s/h)_0$[d]
1	 2a	2.5	220	>99	92	48	2.3
2	 2b	2.5	140	>99	72	42	1.9
3	 2c	2.5	1800	>99	57	36	1.5
4	 2d	2.5	380	>99	49	33	0.58
5	 2e	2.5	340	>99	30	23	No hydrolysis
6	 2f	2.5	320	>99	19	16	0.082
7	 2g	2.5	340	>99	60	38	No hydrolysis
8	 2h	2.5	300	>99	37	26	0.13

[a] Reaction time at maximum DL-phenylalanine conversion.
[b] Determined by chiral HPLC.
[c] Deduced from the ee of the substrate (ee_s) and the product (ee_p): $c = ee_s/(ee_s + ee_p)$.
[d] Determined as a ratio of initial rates for accumulation of both reaction products.

acetamide and L-phenylalanine) must diffuse into the porous structure, in which the reaction was taken place and the product (*N*-phenylacetyl-L-phenylalanine) was generated, thus concentration gradients of both reactants and product were produced and consequently equilibrium was reversed to hydrolysis. Moreover, hydrolysis of *N*-phenylacetyl-L-phenylalanine and phenylacetamide generated phenylacetic acid which induced a pH gradient in the carrier. Since the pH affected the reaction equilibrium as well as the ionization of α-amino functionality of phenylalanine, the pH gradient amplified the general effects of the concentration gradient. Higher pH and temperature increased the solubility of phenylacetic acid and N-phenylacetyl-L-phenylalanine in aqueous medium, and thus diminished their accumulation in the carrier beads of the immobilized PGA. In that way, s/h ratio was improved and higher ee_s and conversion of substrate were achieved. Interestingly, this explanation offered support for choosing *A. faecalis* PGA, which characterized with the broader pH stability range and higher thermostability, as the biocatalyst for the acylation reaction.

Acyl donor is another important factor which influences the acylation reaction rate and enantioselectivity. In this work, 4 pairs of phenylacetic derivatives (**2a** and **2b**, 2c and **2d**, 2e and **2f**, 2g and **2h**) were used as acyl donors in order to provide general guidelines for the choice of proper acyl donors in enantioselective acylation in aqueous media. Each pair included an amide and an ester which bore the same phenylacetic structure and generated the same synthetic and hydrolytic products in the reaction. For instance, the synthetic and hydrolytic products of acyl donors phenylacetamide

Table 3
A. faecalis PGA catalyzed acylation of phenylalanine derivatives



1a-1f L-(3a-3f) D-(1a-1f)

Entry	R	t[a] (min)	c[b] (%)	$(s/h)_0$[c]	Product [L-(**3a–f**)]		Substrate [D-(**1a–f**)]		[D-(**1a–f**)] ee[f] (%)
					Yield[d] (%)	ee[e] (%)	Yield[d] (%)	ee[e] (%)	
1	H	220	48	2.3	90	>99	68	92	98
2	OH	240	47	1.6	92	>99	65	88	92
3	CH_3	220	48	1.8	91	99	64	91	95
4	Cl	220	49	2.9	85	99	73	94	>99
5	F	180	49	2.6	88	99	69	94	99
6	NO_2	340	49	2.7	95	>99	69	94	>99

[a] Reaction time at maximum DL-phenylalanine conversion.
[b] Deduced from the ee of the substrate (ee_s) and the product (ee_p): $c = ee_s/(ee_s + ee_p)$.
[c] Determined as a ratio of initial rates for accumulation of both reaction products.
[d] Yields were calculated taking into account the percentage of conversion c.
[e] Determined by chiral HPLC.
[f] After recrystallization from water/ethanol.

(**2a**) and methyl phenylacetate (**2b**) were *N*-phenylacetyl-L-phenylalanine and phenylacetic acid, respectively. Under the optimized pH and temperature, phenylacetamide gave a conversion and ee_s of 48.7% and 91.6%, respectively (Table 2, entry 1), both of which were the highest among all the acyl donors. Thus resulted the best acyl donor for acylation in aqueous medium, followed by methyl phenylacetate (Table 2, entry 2). Alessandra and co-workers reported that the 4-hydroxyphenylacetic group was more preferentially accepted than phenylacetic group by PGA from *E. coli* when acylated L-tyrosine ethyl ester in toluene.[19] However, the result of this article was otherwise. Both 4-hydroxyphenylacetamide (**2c**) and methyl 4-hydroxyphenylacetate (**2d**) gave lower ee_s, conversion and s/h ratio even with longer reaction time than phenylacetamide and methyl phenylacetate (Table 2, entries 3 and 4). That maybe because of the lack of a Ser residue at the bottom of acyl-binding pocket rendered *A. faecalis* PGA less specific to 4-hydroxyphenylacetic acid derivatives than *E. coli* PGA.[24] The presence of a hydroxyl or amino group at α position of phenylacetamide and methyl phenylacetate (**2e**–**h**) caused an appreciable reduction of the reaction rate and enantioselectivity (Table 2, entries 5–8). Similar results were also reported in Alessandra and co-workers' work, in which they indicated that this lack of activity might be ascribed to unfavorable interactions between the polar amino group of phenyglycine and aromatic side-chains linking the PGA hydrophobic pocket hosting the phenylacetic moiety.[19] Another reason for ineffective acylation of DL-phenylalanine when using (*S*)-methyl mandelate (**2f**) and (*S*)-methyl phenylglycine (**2h**) as acyl donors was the fast hydrolysis of acyl donors, involving self-hydrolysis in alkaline condition and hydrolysis catalyzed by PGA. Interestingly, when (*S*)-methyl mandelate (**2f**) and (*S*)-methyl phenylglycine (**2h**) were replaced by their corresponding amides (**2e** and **2g**), no hydrolysis products (namely (*S*)-mandelic acid and (*S*)-phenyglycine) were detected, which meant neither acyl donors nor acylation products were hydrolyzed by PGA in such conditions. However, both 2e and **2g** needed quite a long time to reach the maximum conversion. Furthermore, the same as (*S*)-methyl phenylglycine, (*S*)-phenylglycinamide can also be accepted as nucleophile by PGA in aqueous medium thereby self-acylation was observed when using them as acyl donors. So neither (*S*)-mandelamide nor (*S*)-phenylglycinamide was an ideal acyl donor for acylation of DL-phenylalanine in aqueous medium despite of their remarkable s/h ratio.

Enantioselective acylation of various racemic *p*-substituted phenylalanines was also investigated (Table 3). *A. faecalis* PGA selectively acylated L-isomer of *p*-substituted phenylalanines from their corresponding racemates in high ee (>99%), leaving the remaining D-*p*-substituted phenylalanines in moderate to high ee (88–94%) (Table 3, entries 1–5). Slight decrease in both conversion and ee_s were observed when phenylalanine was substituted by electron-donating groups at the *p*-position of benzene ring (Table 3, entries 1 and 2), which was due to the fact that *N*-phenylacetyl-4-methyl-L-phenylalanine and *N*-phenylacetyl-L-tyrosine hydrated faster than *N*-phenylacetyl-L-phenylalanine. This could be testified by the lower s/h ratio compared with DL-phenylalaine. On the other hand, both conversion and ee_s were improved when electron-withdrawing groups substituted at the *p*-position of benzene ring (Table 3, entries 3–5). It should be noted that these enantiomeric excess values could be improved after recrystallization from water/ethanol. Thus, as shown in the last column of Table 3, D-amino acids were finally obtained with ee >91%, 4-chloro-D-phenylalanine and 4-nitro-D-phenylalanine were obtained in enantiopure.

In conclusion, we have developed a facile and efficient *A. faecalis* PGA catalyzed enantioselective acylation method in aqueous medium for obtaining pharmacologically interesting D-phenylalanine and its *p*-substituted derivatives. This process has potential as an alternative to other method in industrial applications.

Acknowledgments

This work was supported by the Fundamental Research Funds for the Central Universities, the National Basic Research Program (973 Program) of China (No. 2007CB714300) and the Specialized Research Fund for the Doctoral Program (New Teachers) of Higher Eduḍcation (No. 20090074120015).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.08.056.

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