UNNATURAL D-AMINO ACIDS AS BUILDING BLOCKS OF NEW PEPTIDOMIMETICS

GNIEWOMIR LATACZ, ELŻBIETA PĘKALA, AGNIESZKA CIOPIŃSKA and KATARZYNA KIEĆ-KONONOWICZ

Medical College of Jagiellonian University, Department of Technology and Biotechnology of Drugs, ul. Medyczna 9, 30-688 Kraków, Poland

Compared to native peptides peptidomimetics offers many advantages as newly designed drugs. The benefits include enhanced stability to proteolytic enzymes, better transport through cellular membranes, increased bio-availability, changing agonist to antagonists potency and higher affinity in ligand-receptors interactions. D-amino acids are used to design and obtain new peptidomimetics by their incorporation in place of the natural L-amino acids, either at a specific position, or throughout the whole peptide. D-phenylalanine is one of the preferred amino acids. Addition of hydrophobic phenyl ring results in better transport properties of peptidomimetics through cellular membranes and better enzymatic stability (1, 2).

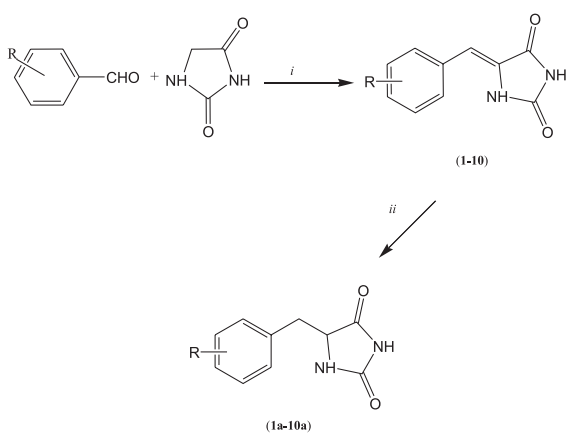
Up to now D-phenylanine and derivatives have been successfully used in design of new efficient peptidomimetics. 4-fluoro-D-phenylalanine derivative implicate in new agonist of melanocortin receptors (MCRs). A common structural feature present in all endogenous ligands (MSH, ACTH) of the melanocortin receptors (MCRs) is His-Phe-Arg-Trp sequence, which has been identified as the minimal peptide fragment necessary for activating the receptors. Further structural modifications toward α-MSH showed that inverting Phe to the D-configuration resulted in an analog with enhanced potency and enzymatic stability. Replacement of the phenyl ring of D-Phe side chain in one of His-D-Phe-Arg-Trp analogue with 4-fluorophenyl group led to potent MCRs agonist with enhanced selectivity (3).

Synthetic decapeptides, Cetrorelix and Abarelix belong to third-generation of modified gonadotropin releasing hormone (GnRH) antagonists and contain in their structures D-4-chlorophenylalanine. GnRH, a linear decapeptide synthesized by gonadotropic cells of hypothalamus, is a central mediator of the human reproductive axis. Cetrorelix and Abarelix reveal high antagonistic activity against naturally occurring GnRH and are used for treatment of sex hormone-dependent disorders (4, 5).

Another important example of peptidomimetic with D-phenylalanine presence is Octreotide – a synthetic somatostatin analogue with similar but more prolonged pharmacological effects. Octreotide is used to treat acromegaly and also to reduce flushing episodes and watery diarrhoea caused by cancerous tumors (carcinoid syndrome) (6).

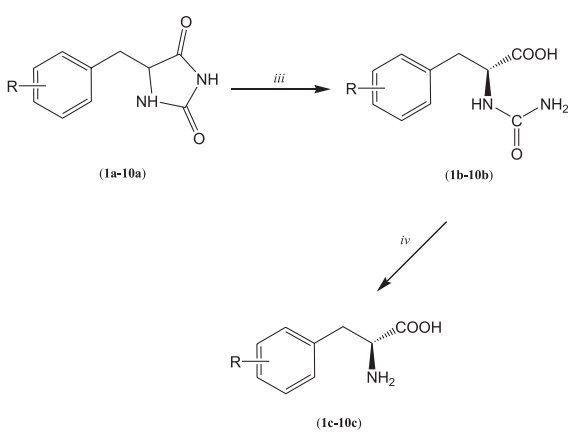
Furthermore, D-amino acid–containing peptides are especially important as pharmaceuticals due to their strong antibacterial activity. Many of them (Bacitracin A, Fungisporin, Gramicidin S, Tyrocidine, Polymyxin,) contain D-phenylalanine (2).

In the present work we report the application of hydantoinase method to obtain D-phenylalanine derivatives. Hydantoinase method consist of three steps:



Reagents and conditions: $i_{(1\text{-}6)}$ = CH_3COOH, CH_3COONa, 5h reflux
$i_{(7\text{-}10)}$ = CH_3COONH_4, mw (450 W)
$ii_{(1a\text{-}10a)}$ = 57% HI, red P, 1h reflux

Figure 1.



Reagents and conditions: $iii_{(1b\text{-}10b)}$ = D-Hyd, borate buffer, 40°C
$iv_{(1c\text{-}10c)}$ = $NaNO_2$, HCl, 0°C

Figure 2.

1. chemical and/or enzymatic racemization of D,L-5-substituted hydantoin

2. opening of the hydantoin ring to the N-carbamoyl-D-amino acid by D-hydantoinase (E.C.3.5.2.2) (6).

3. converting of the N-carbamoyl-D-amino acid to the D-amino acid by diazotization or by the next enzymatic reaction catalyzed by N-carbamylase (E.C.3.5.1) (7).

In our research phenyl ring substituted 5-benzylhydantoins were obtained in racemic form from cheap starting materials such as benzylaldehyde derivatives and hydantoin using first Knoevenagel condensation and next the reduction of unsaturated bond in 5-position of the hydantoin (Figure 1). Obtained ring substituted 5-benzylhydantoin derivatives were used as substrates for immobilized, recombinant from *Escherichia coli* D-hydantoinase. The enzyme catalyzed the enantioselective hydrolytic ring-opening of racemic hydantoin to form N-carbamoyl-D-amino acid, which was next converted by diazotization to the corresponding enantiomerically pure D-amino acid (Figure 2).

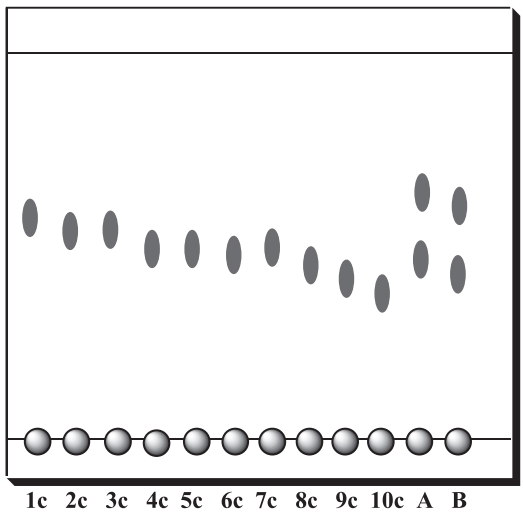
Table 1. 5-Arylidenehydantoin (**1-10**) and 5-benzylhydantoin (**1a-10a**) derivatives.

Comp.	R	Yield [%]	m. p. [OC]	Molecular mass formula	$R_{f\,A}$
1	2-F	65	222-224	206,17 $C_{10}H_7FN_2O_2$	0,46
1a	2-F	90	191-192	208,19 $C_{10}H_9FN_2O_2$	0,28
2	3-F	90	210-212	206,17 $C_{10}H_7FN_2O_2$	0,51
2a	3-F	92	192	208,19 $C_{10}H_9FN_2O_2$	0,21
3	4-F	86	243-250	206,17 $C_{10}H_7FN_2O_2$	0,49
3a	4-F	94	200-201	208,19 $C_{10}H_9FN_2O_2$	0,19
4	2-Br	33,8	300-302	267,08 $C_{10}H_7BrN_2O_2$	0,46
4a	2-Br	80	252-253	269,09 $C_{10}H_9BrN_2O_2$	0,30
5	3-Br	65	235-237	267,08 $C_{10}H_7BrN_2O_2$	0,60
5a	3-Br	91	193-194	269,09 $C_{10}H_9BrN_2O_2$	0,29
6	4-Br	77	307-309	267,08 $C_{10}H_7BrN_2O_2$	0,55
6a	4-Br	88,5	199-200	269,09 $C_{10}H_9BrN_2O_2$	0,23
7	2-CH_3	69,3	199-201	202,21 $C_{11}H_{10}N_2O_2$	0,69
7a	2-CH_3	63,7	175-177	204,23 $C_{11}H_{10}N_2O_2$	0,37
8	3-CH_3	44,5	226-228	202,21 $C_{11}H_{10}N_2O_2$	0,70
8a	3-CH_3	49,5	184-186	204,23 $C_{11}H_{10}N_2O_2$	0,38
9	4-CH_3	61,9	263-266	202,21 $C_{11}H_{10}N_2O_2$	0,69
9a	4-CH_3	58,8	208-210	204,23 $C_{11}H_{10}N_2O_2$	0,38
10	2,4-CH_3	60,2	183-185	216,24 $C_{12}H_{12}N_2O_2$	0,68
10a	2,4-CH_3	18,4	215-217	218,25 $C_{12}H_{14}N_2O_2$	0,41

A = chloroform/ethyl acetate (1:1, v/v)

Table 2. IR and ^{1}H NMR spectral data of compounds **1a-10a**.

No.	I R (KBr)[cm-1]			1H NMR δ (ppm)
	NH	C=O (2)	C=O (4)	
1a	3184	1733	1781	2,85-2,90 (dd, *J*=6,4 Hz,1H, H -C-H); 3,00-3,05 (dd, *J*=4,9 Hz, 1H, H-C-H); 4,29 (t, *J*=5,6 Hz, 1H, CH); 7,12 (q, *J*=7,6Hz, 2H, Ph-3-H, Ph-6-H); 7,26 (t, *J*=6,9 Hz, 2H, Ph-4-H, Ph-5-H); 7,92 (s, 1H, N_1H); 10,52 (s, 1H, N_3H);
2a	3179	1734	1780	2,93 (qu, *J*=7,1 Hz, 2H, CH_2); 4,34 (t, *J*=5,2 Hz, 1H, CH); 6,99-7,07 (m, 3H, Ph-2-H, Ph-4-H, Ph-6-H); 7,31(q, *J*=7,4 Hz, 1H, Ph-5-H); 7,91(s, 1H, N_1H); 10,47 (s, 1H, N_3H);
3a	3245	1708	1757	2,96 (d, *J*=4,9 Hz, 2H, CH_2); 4,30 (t, *J*=4,9 Hz, 1H, CH); 7,06-7,21 (m, 4H, Ph-2-H, Ph-3-H, Ph-5-H, Ph-6-H); 7,89 (s, 1H, N_1H); 10,42 (s, 1H, N_3H);
4a	3187	1731	1783	2,88-2,96 (dd, *J*=9,0 Hz, 1H, H -C-H); 3,16-3,22 (dd, *J*=5,0 Hz, 1H, H-C-H); 4,32 (t, *J*=5,0 Hz, 1H, CH); 7,17 – 7,24 (m, 1H, Ph-6-H); 7, 30 – 7, 35 (m, 2H, Ph-4-H, Ph-5-H); 7, 59 (d, *J*=7,0Hz, 1H, Ph-3-H); 7,91 (s, 1H, N_1H); 10,67 (s, 1H, N_3H);
5a	3239	1700	1756 2.94	(t, *J*= 5,-Hz, 2H, CH_2); 4,36 (t, *J*=5,0 Hz, 1H, CH); 7,13-7,18 (m, 1H, Ph-2-H); 7,26-7,35 (m, 4H, Ph-4-H, Ph-5-H, Ph-6-H), 7,93 (s, 1H, N_1H); 10,72 (s, 1H, N_3H);
6a	3241	1706	1756	2,89(q, *J*=10,0 Hz, 2H, CH_2); 3,34 (t, *J*=10,0 Hz, 1H, CH); 7,35 (d, *J*=8,0 Hz 2H, Ph-2-H, Ph-6-H); 7,48 (d, *J*=8,0 Hz, 2H, Ph-3-H, Ph-5-H); 7,94 (s, 1H, N_1H); 10,48 (s, 1H, N_3H);
7a	3185	1730	1784	2,26 (s, 3H,CH_3);2,80-2,87 (dd, *J*=14,4 Hz, 1H, H -C-H); 2,96-3.02 (dd, *J*=14,4 Hz, 1H, H -C-H); 4,28 (m, 1H, CH); 7,06 (m, 4H, Ph-2-H, Ph-3-H,Ph-4-H, Ph-5-H); 7,89(s, 1H, N_1H); 10, 52(s, 1H, N_3H);
8a	3225	1728	1782	2,24(s, 3H, CH_3); 2,79-2,92(m, 2H, CH_2); 4,27(t, *J*=4,6 Hz, 1H, CH); 6,95-7,03(m, 3H, Ph-2-H, Ph-4-H, Ph-6-H); 7,14(t, *J*=7,4 Hz, 1H, Ph-5-H); 7,89(s, 1H, N_1H); 10, 41(s, 1H, N_3H);
9a	3246	1706	1755	2,24(s, 3H, CH_3); 2,85-2,86(d, *J*=4,9 Hz, 2H, CH_2); 4,25-4,29(m, 1H, CH); 7,05(s, 4H, Ph-2-H, Ph-3-H, Ph-5-H, Ph-6-H); 7,86(s, 1H, N_1H); 10, 36(s, 1H, N_3H);
10a	3221	1730	1779	2,20 (s, 3H,CH_3); 2,22 (s, 3H,CH_3);2,76-2,83 (dd, 14,4 Hz 1H, H -C-H); 2,90-2,97 (dd, *J*=14,4 Hz) 1H, H -C-H); 4,23-4,37 (m, 1H, CH); 6,87-6,93 (m, 2H, Ph-4-H, Ph-5-H); 7,00 (d, *J*=7,7 Hz, 1H, Ph-2-H); 7,86(s, 1H, N_1H); 10, 45 (s, 1H, N_3H);



A= D,L-4-fluorophenylalanine
B= D,L-4-chlorophenylalanine
C: methanol/water/acetonitryle (50:50:200)

Figure 3. The image shows chiral TLC confirmation of the presence of D-amino acids.

EXPERIMENTAL

Chemicals and reagents

Hydantoin, benzylaldehyde derivatives, red phosphorus, sodium acetate, ammonium acetate and solvents which were used in chemical synthesis of 5-benzylhydantoin derivatives were obtained from commercial suppliers and used without further purification. Biotransfomations were carried out in borate buffer pH = 9 (Merck) with commercially available D-hydantoinase (E.C 3.5.2.2), immobilized, cloned and expressed in *Escherichia coli*, (Fluka, 53,1 U/g activity).

General methods

All melting points were measured in glass capillary tubes using Mel-Temp® Laboratory Devices Inc. USA apparatus and are uncorrected. ^{1}H-NMR spectra were recorded on Varian Mercury-VX instrument at 300 MHz using TMS as an internal standard in DMSO-d_6. IR spectra were recorded in KBr pellets using a Jasco FT/IR-410 spectrophotometer. Reactions under microwave irradiation were performed using Samsung M1618 domestic microwave oven.

Analysis by TLC and Chiral TLC

The reactions were monitored by thin-layer chromatography using commercially available TLC aluminium sheets (silica gel 60 F_{254} Merck) using A: chloroform/ethyl acetate (1:1, v/v), B: butanol/acetic acid/water (4:1:1, v/v/v) eluents and visualized by UV light (254 nm). The enantiomeric purities of D-amino acids were determined using chiral thin layer chromatography. The samples were applied to Chiralplate® Macherey-Nagel, 0.25 mm silica gel, amino acids were eluted with C: methanol/water/acetonitrile (50:50:200, v/v/v). Spots were visualized after spraying with 0,3% ninhydrin and heating at 100°C for 3 – 5 min.

Synthesis of racemic phenyl ring-substituted 5-benzylhydantoins

5-Arylidenehydantoins (**1-6**) were synthesized according to Knoevenagel procedure. A mixture of 0.05 mol (5.05 g) of hydantoin, 0,05 mol of appropriate benzaldehyde, 0.2 mol (16.7 g) of anhydrous sodium acetate and 100 mL of 99,5% acetic acid was stirred and refluxed for 5 h. The mixture was allowed to stand overnight at ambient temperature. The precipitated crystalline material was filtered off, washed with water and after drying recrystallized from acetic acid. The reaction was monitored by TLC with A: chloroform/ethyl acetate (1:1, v/v).

5-Arylidenehydantoins (**7-10**) were synthesized by condensation of 0.01 mol (1.01 g) of hydantoin and 0.01 mol of appropriate benzaldehyde which was carried out under microwave irradiation in the presence of 0.01 mol (0.77 g) of ammonia acetate at 450 W of power for 1 min, and was continued after cooling for 0.5 min. The crude 5-arylidenehydantoin was suspended in 30 mL of 99.5% acetic acid and refluxed for 0.5 h. The solution was cooled and allowed to stand at 4°C overnight. The precipitated crystalline material was filtered off and washed with water.

5-Benzylhydantoins (**1a-10a**) were synthesized by reduction of 0.005 mol of appropriate 5-arylidenehydantoin (**1-10**) with 4.5 mL of 57% hydroiodic acid in the presence of 1 g of red phosphorus. The mixture was heated under reflux for 0.5 h. Afterward 30 mL of distilled water was added and the precipitate was filtered off (8).

Physicochemical properties

The obtained compounds (**1-10**) and (**1a-10a**) are presented in Table 1. The IR and ^{1}H NMR spectral data of compounds (**1a-10a**) are presented in Table 2. Compounds **(1-10)** were described previously by Thenmozhiyal et al. (9). Compounds **7** and **7a** were obtained and described by Hosztafi et al. (8). Compound **9** was obtained by Tan et al. (10).

Biotransformation of phenyl ring-substituted 5-benzylohydantoins

0,125 mmol of 5-benzylhydantoin (**1a-10a**) was dissolved in 0.5 mL DMSO and added to 15 mL of buffer solution (H_3BO_3/HCl/NaOH, pH = 9). In this mixture 0,3 g D-hydantoinase (53.1 U/g) was suspended. The incubation was kept at 40°C for 72 h. The process was monitored by TLC with B: butanol/acetic acid/water (4:1:1, v/v/v). Spots were visualized by UV light (254 nm). The obtained N-carbamoyl-D-amino acids (**1b-10b**) were not isolated (Figure 2).

Diazotization

The reaction mixture was acidified with 3.5 M HCl to pH = 2-3. N-carbamoyl-D-amino acids (**1b-10b**) were converted into the corresponding D-amino acids (**1c-10c**) by means of diazotization – 140 µL of 3.5 M $NaNO_2$ solution was added and stored in 0°C for 6 h.

Identification

The enantiomeric purities of D-amino acids were determined using the chiral thin layer chromatography in the presence of commercial D,L-4-fluorophenylalanine (Nutritional Biochem. Corp.) and D,L-p-chlorophenylalanine (Sigma). The samples were applied to Chiralplate® Macherey-Nagel, 0.25 mm silica gel and were eluted with C: methanol/water/acetonitrile (50:50:200, v/v/v). Amino acids were detected after spraying with 0.3% ninhydrin and heating at 100°C for 3-5 min (Figure 3).

CONCLUSIONS

Using hydantoinase method enantiomerically pure D-phenylalanine derivatives (**1c-10c**) were obtained. Chiral TLC confirmed the presence of D-enantiomer in the reaction mixture in high optical yield (Figure 3). Obtained D-amino acids were not recovered from the reaction mixture thus the reaction efficiency of hydantoinase method was not examined. The presence of little amounts of unreacted 5-benzylhydantoins (**1a-10a**) and N-carbamoyl-D-amino acids (**1b-10b**) in reaction mixture were observed by TLC. However, all tested phenyl ring-substituted 5-benzylohydantoins (**1a-10a**) were tolerated as substrates by immobilized, cloned and expressed in *Escherichia coli* D-hydantoinase (Fluka). Condensation of compounds (**7-10**) was carried out under microwave irradiation. Reactions were performed using domestic microwave oven and gave several advantages: easy work-up after the reaction, reduction of the by-products formed in usual thermal degradation and short reaction time compared to conventional heating.

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