BIOORGANIC CHEMISTRY 17, 275–280 (1989)

Resolution of Racemic Amino Acids with Thermitase

LESZEK ŁANKIEWICZ,* FRANCISZEK KASPRZYKOWSKI,*
ZBIGNIEW GRZONKA,* URSULA KETTMANN,† AND PETER HERMANN†

*Institute of Chemistry, University of Gdansk, 80–952 Gdansk, Poland, and †Institut für Biochemie, Bereich Medizin, Martin-Luther-Universität, 4010 Halle(Saale), German Democratic Republic

Received August 24, 1988

Racemic *t*-butyloxycarbonyl-amino acids methyl esters [*t*-butyloxycarbonyl-(4-chloro)phenylalanine methyl ester, *t*-butyloxycarbonyl-(2,4-dichloro)phenylalanine methyl ester, *t*-butyloxycarbonyl-(3,4-dichloro)phenylalanine methyl ester, *t*-butyloxycarbonyl-(2-naphthyl)alanine methyl ester, and *t*-butyloxycarbonyl-(*S*-benzyl)cysteine methyl ester] were resolved by asymmetric hydrolysis with thermitase.

INTRODUCTION

Unusual amino acids are widely used in the syntheses of analogs of biologically active peptides (*1*). In particular, they are very useful in the design of peptide hormone antagonists and enzyme inhibitors. In the synthesis of amino acids of specific configuration the most frequent methods used for the resolution of racemic compounds are (i) the diastereoisomeric salts formation method, and (ii) the enzymatic method. The last method usually requires a preparation of a specific amino acid derivative, e.g., *N*-acyl derivative, which after resolution cannot be used directly in peptide synthesis. In such a case the resolved stereoisomer needs further chemical treatments, which can change the optical purity of the compound.

Thermitase, an alkaline serine protease from *Thermoactinomyces* (*2*, *3*), is characterized by a high esterase/peptidase activity ratio. Therefore, thermitase was found to be a useful reagent in the selective stereospecific hydrolysis of C-terminal ester bonds of peptides (*4*). We now report the use of thermitase for the resolution of racemic *t*-butyloxycarbonyl-amino acids, which are the most commonly used N-protected amino acid derivatives in peptide synthesis. The following racemic methyl esters of N-protected amino acids were resolved: **7**, *t*-butyloxycarbonyl-DL-(4-chloro)phenylalanine methyl ester, Boc-DL-Phe(4-Cl)-OMe[1]; **8**, *t*-butyloxycarbonyl-DL-(2,4-dichloro)phenylalanine methyl ester, Boc-DL-Phe(2,4-diCl)-

[1] Abbreviations used: Boc-DL-Phe(4-Cl)-OMe, *t*-butyloxycarbonyl-DL-(4-chloro)phenylalanine methyl ester; Boc-DL-Phe(2,4-diCl)-OMe, *t*-butyloxycarbonyl-DL-(2,4-dichloro)phenylalanine methyl ester; Boc-DL-Phe(3,4-diCl)-OMe, *t*-butyloxycarbonyl-DL-(3,4-dichloro)phenylalanine methyl ester; Boc-DL-Nal(2)-OMe, *t*-butyloxycarbonyl-DL-(2-naphthyl)alanine methyl ester; Boc-DL-Cys(Bzl)-OMe, *t*-butyloxycarbonyl-(*S*-benzyl)cysteine methyl ester; DMF, dimethylformamide.

0045-2068/89 $3.00

OMe; **9**, *t*-butyloxycarbonyl-DL-(3,4-dichloro)phenylalanine methyl ester, Boc-DL-Phe(3,4-diCl)-OMe; **10**, *t*-butyloxycarbonyl-DL-(2-naphthyl)alanine methyl ester, Boc-DL-Nal(2)-OMe; and **11**, *t*-butyloxycarbonyl-(*S*-benzyl)cysteine methyl ester, Boc-DL-Cys(Bzl)-OMe.

RESULTS AND DISCUSSION

Racemic amino acids containing aromatic substituents were synthesized by the condensation of the appropriate aromatic methylhalide with diethyl acetamidomalonate (*5*). The alkylated malonates were hydrolyzed and decarboxylated in boiling 6 N hydrochloric acid to give the racemic amino acids. Racemic *S*-benzylcysteine was obtained according to our published procedure (*6*). By means of the subsequent esterification (methanol, thionyl chloride) (*7*), followed by the formation of *N-tert*-butyloxycarbonyl derivatives (di-*tert* butyl dicarbonate, triethylamine, tetrahydrofuran) (*8*), the substrates for the enzymatic resolution were formed (Table 1).

Amino acid derivatives **7–11** were resolved by asymmetric hydrolysis with thermitase. The racemic substrates, because of their low solubility in water, were resolved in aqueous dimethylformamide. Generally, the 25–30% DMF was used as a solvent. The resolution was carried out at 55°C in a reaction vessel connected to an automatic titrator until base uptake ceased. The time required for the hydrolysis of L-enantiomer derivative depended on the steric effect of the amino acid side chain. It varied from 6 h for cysteine derivative up to 42 h for 2,4-dichlorophenylalanine. It should be noticed that in the case of Boc-DL-Phe(2,6-

TABLE 1

Physical Constants of Racemic Amino Acid Derivatives

Derivative	Yield (%)	mp (°C)	Formula[a] (molecular weight)
Amino acid methyl esters hydrochlorides			
3 HCl·H-Phe(4-Cl)-OMe	95.4	177–178	$C_{10}H_{13}NO_2Cl_2$ (250.1)
4 HCl·H-Phe(2,4-diCl)-OMe	93.2	185–186	$C_{10}H_{12}NO_2Cl_3$ (284.6)
5 HCl·H-Phe(3,4-diCl)-OMe	94.8	156–158	$C_{10}H_{12}NO_2Cl_3$ (284.6)
6 HCl·H-Nal(2)-OMe	90.5	182–183	$C_{14}H_{16}NO_2Cl$ (265.7)
t-Butyloxycarbonyl–amino acid methyl esters			
7 Boc-Phe(4-Cl)-OMe	95.7	79–80	$C_{15}H_{20}NO_4Cl$ (313.8)
8 Boc-Phe(2,4-diCl)-OMe	96.3	105–106	$C_{15}H_{19}NO_4Cl_2$ (348.2)
9 Boc-Phe(3,4-diCl)-OMe	98.1	97–99	$C_{15}H_{19}NO_4Cl_2$ (348.2)
10 Boc-Nal(2)-OMe	93.2	92–93	$C_{19}H_{23}NO_4$ (329.4)
11 Boc-Cys(Bzl)-OMe[b]	95.0	43–45	$C_{16}H_{23}NO_4S$ (325.4)

[a] The analytical results indicated by the elemental symbols were ±0.4% of the theoretical values, and molecular weights determined by MS-FD confirmed the calculated values.

[b] Obtained from racemic *S*-benzyl-cysteine through *t*-butyloxycarbonyl-derivative [Boc-DL-Cys(Bzl)-OH; mp 110–112°C] by esterification using diazomethane.

TABLE 2

Results of Enzymatic Hydrolysis of Racemic Methyl Esters of *t*-Butyloxycarbonyl-Amino Acids Using Thermitase

Derivative	Yield[a] (%)	mp (°C)	$[\alpha]_D^{21}$ (deg.)
t-Butyloxycarbonyl-L-amino acids			
12 Boc-L-Phe(4-Cl)-OH	92.7	111–113	+14.6 (*c* = 1, MeOH)[b]
			+22.4 (*c* = 1, EtOH)[c]
13 Boc-L-Phe(2,4-diCl)-OH	93.3	155–157	−21.4 (*c* = 1, MeOH)
			−24.9 (*c* = 1, AcOEt)[d]
14 Boc-L-Phe(3,4-diCl)-OH	94.4	119–120	+16.2 (*c* = 1, MeOH)
			+24.1 (*c* = 1, AcOEt)[e]
15 Boc-L-Nal(2)-OH	91.6	92–94	+46.1 (*c* = 1, MeOH)
			+43.7 (*c* = 1, EtOH)[f]
16 Boc-L-Cys(Bzl)-OH	85.0	64–65	−44.3 (*c* = 1, AcOH)[g]
t-Butyloxycarbonyl-D-amino acid methyl esters			
17 Boc-D-Phe(4-Cl)-OMe	95.5	75–76	+7.6 (*c* = 1, MeOH)
18 Boc-D-Phe(2,4-diCl)-OMe	97.5	85–86	+19.1 (*c* = 1, MeOH)
19 Boc-D-Phe(3,4-diCl)-OMe	94.6	59–60	+4.1 (*c* = 1, MeOH)
20 Boc-D-Nal(2)-OMe	80.1	84–85	−13.1 (*c* = 1, MeOH)
21 Boc-D-Cys(Bzl)-OMe	97.0	49–51	+35.3 (*c* = 1, MeOH)

[a] Based on theoretical contents of L- or D-enantiomer in the racemic mixture.
[b] Ref. (*9*): +14.4.
[c] Ref. (*10*): +20.6.
[d] Ref. (*10*): +25.3.
[e] Ref. (*10*): +22.0.
[f] Ref. (*10*): +42.1.
[g] Ref. (*11*): −41.0.

diCl)-OMe the enzymatic hydrolysis was so slow that typical alkaline hydrolysis took place and only insufficient resolution was observed. The quantitative half-time rates of hydrolysis were not calculated from the experimental curves because of differences in the reaction conditions (e.g., fresh amounts of thermitase were added during hydrolysis in the case of slowly hydrolyzing amino acid derivatives). However, it was possible to ascertain that the hydrolysis rate decreases in the following order: Boc-DL-Cys(Bzl)-OMe (**11**) > Boc-DL-Phe(4-Cl)-OMe (**7**) > Boc-DL-Nal(2)-OMe (**10**) > Boc-DL-Phe(3,4-diCl)-OMe (**9**) > Boc-DL-Phe(2,4-diCl)-OMe (**8**).

After resolution the reaction mixture was diluted with water and the precipitated unhydrolyzed D-enantiomer was isolated. Acidification of the filtrate gave the desired L-enantiomer of Boc-amino acid. Table 2 summarizes the results of the enzymatic resolution of racemic amino acid derivatives with thermitase.

Porter *et al.* (*10*) reported recently the synthesis of some ring-substituted optically active *t*-butyloxycarbonyl-phenylalanines and tryptophanes. In this work they described the resolution of racemic *N*-acetyl- or *N*-trifluoroacetyl-amino acids with chymotrypsin or carboxypeptidase A, respectively. The resolved amino acid derivatives were then hydrolyzed and finally *t*-butyloxycarbonylated.

In conclusion, we have developed a synthetic method which can be used for the direct preparation of L- and D-stereoisomers of Boc-amino acids for peptide synthesis.

EXPERIMENTAL PROCEDURES

Instrumentation. Melting points are uncorrected. Optical rotation was measured on a Hilger-Watts polarimeter with an accuracy of 0.01°. Infrared spectra (Carl Zeiss 71IR spectrophotometer) and ^1H NMR spectra (80 MHz BS 487 Tesla spectrometer) confirmed the structures of the synthesized compounds. The elemental analytical results determined on a Carlo Erba Model 1106 analyzer indicated by the elemental symbols were +0.4% of their theoretical values. Molecular ions were determined on a Varian MAT-711 mass spectrometer by the field desorption technique. TLC was performed on silica gel plates (Merck) in the following solvent systems: (A) chloroform–methanol (9 : 1, v/v); (B) benzene–acetone (3 : 1, v/v).

Enzyme. Thermitase was isolated from the culture medium of *Thermoactinomyces vulgaris* by a single step adsorption procedure using porous glass CPG-10 (2). The enzyme solution (5.5 mg/ml) was stored at +4°C in 0.1 M ammonium acetate buffer (pH 6.0) under nitrogen. Proteolytic activity determined photometrically at pH 8.0 and 55°C in 25 mM Tris · HCl buffer, using Suc-$(Ala)_3$-*p*-nitroanalide (2.5×10^{-4} M) as a substrate, was 0.6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

Diethyl acetamido-(4-chlorobenzyl)malonate (*1*). Diethyl acetamidomalonate (21.7 g, 100 mmol), anhydrous K_2CO_3 (12.34 g, 110 mmol), 4-chlorobenzyl chloride (17.71 g, 110 mmol), and 18-crown-6 (0.264 g, 1 mmol) in DMF (50 ml) were heated at 100–110°C for 2 h until acetamidomalonate disappeared in the reaction mixture (TLC) (*5*). The mixture was poured out onto ice, and the solidified product was filtered and washed several times with water and dried. Crystallization from a toluene–hexane mixture gave 30.5 g (89%): mp 140–141°C. Lit. (*10*): mp 143°C.

DL-(4-Chloro)phenylalanine hydrochloride (*2*). A suspension of diethyl acetamido-(4-chlorobenzyl)malonate **1** (29.14 g, 85 mmol) in 250 ml of 6 N hydrochloric acid was refluxed until a clear solution was formed (ca. 5 h). The solvent was evaporated and the product was crystallized from methanol–water. Collection of the amino acid by filtration and drying gave 18.13 g (90%): mp 239–240°C.

DL-(4-Chloro)phenylalanine methyl ester hydrochloride (*3*). A thionyl chloride (7.28 ml) was added to the suspension of amino acid derivative **2** (18.05 g, 76.5 mmol) in methanol (80 ml). After 48 h the solvent was evaporated and the residue was crystallized from methanol–ethyl ether. Yield: 18.25 g (95.4%); mp 177–178°C.

t-Butyloxycarbonyl-DL-(4-chloro)phenylalanine methyl ester (*7*). Compound **3** (17.51 g, 70 mmol) suspended in ethyl ether–*t*-butanol mixture (1 : 1, v/v; 150 ml) was treated at 0°C with triethylamine (9.76 ml, 70 mmol). Then di-*tert*-butyl dicarbonate (16.8 g, 77 mmol) was added and the reaction mixture was stirred for 2 h. Solvents were evaporated and the residue was extracted with ethyl acetate. The

TABLE 3

Results of Alkaline Hydrolysis of Methyl Esters of *N-t*-Butyloxycarbonyl-D-Amino Acids

Derivative	Yield (%)	mp (°C)	$[\alpha]_D^{21}$ (deg) (c = 1, MeOH)
22 Boc-D-Phe(4-Cl)-OH	86.3	110–112	−13.8
23 Boc-D-Phe(2,4-diCl)-OH	87.3	155–157	+21.0
24 Boc-D-Phe(3,4-diCl)-OH	85.4	118–120	−15.6
25 Boc-D-Nal(2)-OH	85.4	92–93	−48.8
26 Boc-D-Cys(Bzl)-OH	70.0	62–64	+43.0[a]

[a] c = 1, AcOH.

AcOEt solution was washed with water, dried over $MgSO_4$, and evaporated. Product was crystallized from AcOEt–hexane: 21.41 g (97.5%); mp 79–80°C.

Resolution of racemic methyl ester of N-t-butyloxycarbonyl-(4-chloro)phenylalanine (**7**) *with thermitase*. An aqueous solution of $CaCl_2$ (1.25×10^{-3} M; 100 ml) was added to the solution of Boc-DL-Phe(4-Cl)-OMe **7** (5 g, 15.95 mmol) in DMF (150 ml) and water (250 ml). The reaction mixture was warmed to 55°C and connected to an automatic titrator (Radiometer, Denmark), delivering 0.5 N NaOH, to maintain a constant pH of 8 during the hydrolysis. A solution of thermitase (1.6 ml; ca. 8 mg of enzyme) was then added and the reaction mixture was stirred until base uptake ceased (ca. 16 h). Water (500 ml) was added and the mixture was kept for 1 h in a refrigerator. Precipitated Boc-D-Phe(4-Cl)-OMe **17** was then filtered, washed with water, and dried to yield 2.39 g (95.5% based on theoretical contents of D-enantiomer) (see Table 2). The combined filtrates were extracted with ethyl ether (3 × 50 ml) and acidified with 2 N HCl to pH 3. The oily product was extracted from the aqueous phase with ethyl ether (5 × 50 ml). The combined organic phase was washed with water, dried over $MgSO_4$, and evaporated to yield 2.21 g (92.7%) of Boc-L-Phe(4-Cl)-OH **12**; mp 111–113°C; $[\alpha]_D^{21}$ +14° (c = 1, MeOH).

t-Butyloxycarbonyl-D-(4-chloro)phenylalanine (**22**). Sodium hydroxide (2 N, 3.5 ml) was added to the solution of Boc-D-Phe(4-Cl)-OMe **17** (2.19 g, 7 mmol) in methanol (15 ml). After 1 h the solution was acidified to pH 3 with 2 N HCl. Ethyl ether extraction (3 × 50 ml) and drying the combined extracts ($MgSO_4$) gave Boc-D-Phe(4-Cl)-OH **22** (1.81 g; 86.3%) after evaporation: mp 110–112°C; $[\alpha]_D^{21}$ −13.8° (c = 1, MeOH) (see Table 3).

ACKNOWLEDGMENTS

This work was supported, in part, by the Polish Academy of Sciences (CPBR-3.13.4.3.2). The authors thank Mrs. Heide Scholz and Dipl. Biochemist Steffi Dudek for technical help.

REFERENCES

1. ROBERTS, D. C., AND VELLACCIO, F. (1983) *in* The Peptides (Gross, E., and Meienhofer, J., Eds.), Vol. 5, pp. 342–449, Academic Press, New York.
2. KLEINE, R., ROTHE, U., KETTMANN, U., AND SCHELLE, H. (1981) *in* Proteinases and Their Inhibitors (Turk, V., and Vitale, L., Eds.), pp. 201–212, Pergamon, Oxford.
3. KLEINE, R. (1982) *Acta Biol Med. Germ.* **41,** 89–102.
4. HERMANN, P., AND SALEWSKI, L. (1983) *in* Peptides 1982 (Blaha, K., and Malon, P., Eds.), pp. 399–402, de Gruyter, Berlin.
5. KOŁODZIEJCZYK, A., AND ARENDT, A. (1980) *Pol. J. Chem.* **54,** 1327–1332.
6. LIBEREK, B., GRZONKA, Z., AND MICHALIK, A. (1966) *Roczniki Chem.* **40,** 683–687.
7. BRENNER, M., AND HUBER, W. (1953) *Helv. Chim. Acta* **36,** 1109–1113.
8. TARBELL, D. S., YAMAMOTO, Y., AND POPE, B. M. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 730–733.
9. PERSEO, G., PIANI, S., AND DE CASTIGLIONI, R. (1983) *Int. J. Peptide Protein Res.* **21,** 227–230.
10. PORTER, J., DYKERT, J., AND RIVIER, J. (1987) *Int. J. Peptide Protein Res.* **30,** 13–21.
11. ANDERSON, G. W., AND MCGREGOR, A. C. (1957) *J. Amer. Chem. Soc.* **79,** 6180–6186.