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Synthesis of achiral and chiral peptide nucleic acid (PNA) monomers using Mitsunobu reaction

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Abstract—Peptide nucleic acids (PNAs) are intensively studied DNA analogues. We elaborated an efficient procedure for the synthesis of *N*-, *C*-protected pseudodipeptides with a reduced peptide bond and then peptide nucleic acid (PNA) monomers, based on the Mitsunobu reaction of *N*-Boc-β-amino alcohols with *N*-*o*-nitrobenzenesulfonyl-protected (*o*NBS-protected) amino acid esters. Using the new procedure, we obtained protected PNA monomer backbones with various amino acid side chains. The pseudodipeptide secondary amine groups were then deprotected by thiolysis, and after appropriate work-up, acylated with thymin-1-ylacetic acid to give the protected monomers. The procedure seems to be of general applicability and allows various modifications of PNA structure by using diverse alcohols and amino acid esters.

1. Introduction

Peptide (or more generally, polyamide) nucleic acids (PNAs) are relatively novel DNA analogues which can mimic oligonucleotides forming heteroduplexes with complementary DNA or RNA.[1] In PNAs a polyamide or peptide backbone replaces the phosphodiester pentose backbone of DNA or RNA. Depending on the manner of the attachment of the nucleobase to the polyamide backbone, two main groups of polyamide nucleic acids can be singled out:[2]

- Type **I**: PNAs containing a polyamide backbone consisting of *N*-(aminoalkyl)aminoacid units to whose secondary amine groups nucleobases are attached by an alkylcarbonyl linker. These PNAs may be achiral or chiral (as illustrated in Fig. 1)
- Type **II**: PNAs containing a backbone consisting of amino acid residues carrying nucleobases in their side chains, frequently called 'chiral PNA' or 'cPNA'.

The most widely known PNAs, those based on a *N*-(2-aminoethyl)glycine backbone (Fig. 1), were designed and synthesised in 1991 by a group of Danish chemists.[1] These PNA molecules efficiently and sequence-specifically bind to the complementary (according to Watson–Crick or Hoogsteen rules) strand of DNA, RNA or PNA oligomers.[3] Complexes of PNA oligomers with natural nucleic acids show high thermal stability and PNAs of this type prove to be better ligands of DNA or RNA than native nucleic acids.[2] PNA oligomers show the ability to displace the pyrimidine strand of homopurine/homopyrimidine dsDNA and to form PNA–DNA duplexes or PNA*DNA–PNA triplexes.[4] Due to their interesting properties, PNAs can serve as e.g. nucleic acid-targeted compounds with antigene and antisense properties or excellent molecular probes, and they already have numerous applications in the fields of molecular biology and experimental medicine.[5] Both types of PNAs may also find various interesting applications in chemistry[6] and technology, e.g. as electrochemical biosensors,[7] and in optical data storage.[8] The main limitations of the usefulness of PNAs are their poor solubility in physiological solutions and low permeability through cellular membranes. The PNA structure is easy to modify and it is probable that synthesis of altered (e.g. chiral)[9] monomers would subsequently result in oligomers with improved

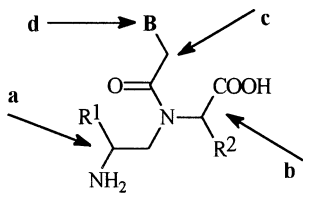

Figure 1. Unprotected type **I** chiral PNA monomer. Conventional parts: aminoalkyl (a), amino acid (b), linker (c), and nucleobase (**B**) (d). R^1 or R^2 (or both) are different from H.

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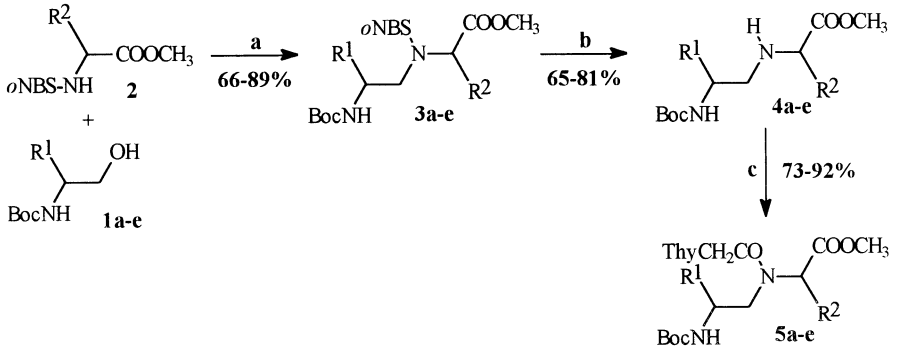


Figure 2. Scheme of the synthesis of PNA monomers. R^1=–H, $–CH_3$, $–CH(CH_3)_2$, $–CH_2CH(CH_3)_2$, $–CH_2C_6H_5$; R^2=H, but the procedure allows to use also other side chains. a—TPP, DEAD in dry THF or TPP, DIAD in dry THF; b—PhSH, K_2CO_3 in CH_3CN; c—ThyAcOH, EDC, HODhbt in DMF.

properties,[2] e.g. with better permeability through cellular membrances[10] or with better solubility in physiological fluids.[9b] Modified PNA oligomers may constitute a substrate for DNA polymerases,[11] and may have numerous other interesting properties.[12]

In typical PNA oligomers the phosphodiester backbone of DNA is replaced by *N*-(2-aminoethyl)glycine units, therefore the synthesis of a protected pseudopeptide with the reduced peptide bond $\psi(CH_2NH)$ most frequently serves as the key step in the synthesis of type **I** PNA monomers. The most common method of reduced peptide bond synthesis is the reductive amination of *N*-protected α-amino aldehydes with amino esters.[13] However, *N*-protected α-aminoaldehydes are both chemically and configurationally unstable and the reductive amination of *N*-protected glycinals gives rise to particular concern due to the chemical instability of glycinals and possible branching of the peptide backbone during the reaction.[14]

In 1996 we elaborated a new method of synthesising PNA monomer backbones[15] or pseudopeptide bond[16] based on the Mitsunobu reaction[17] (see several reviews on the usefulness of the reaction)[18] with *N*-tosyl-protected (Ts) amino acid esters as acidic components of the reaction (Ts derivatives as components of the Mitsunobu reaction were first introduced, for amine alkylation, by Henry et al. and Edwards et al.,[19,20] and then used for *N*-alkylation of amino acids by Papaioannou et al.).[21] As the removal of the Ts group requires harsh conditions, several modifications of the Mitsunobu-type *N*-alkylation procedure have recently been devised, employing 2,2,5,7,8-pentamethylochromanyl-6-sulfonyl (Pmc),[22] *o*- or *p*-nitrobenzenesulfonyl (*o*- or *p*NBS)[23] or *o*,*p*-dinitrobenzenesulfonyl[24] protective groups.

Since our previously proposed procedure requires conditions incompatible with various functional groups, here we present a new, modified and efficient method of the synthesis of various peptidic parts of PNA monomers, employing the Mitsunobu reaction of *N*-*o*NBS-protected amino acid esters and *N*-Boc-protected β-amino alcohols. To the best of our knowledge, the Mitsunobu reaction has already been used in PNA chemistry, but (except our earlier attempts)[15] only in type **II** PNA monomer synthesis—as the key step in the attachment of the nucleobases to the side chains of the hydroxyamino acids or for the inversion of the configuration of carbinol carbon in some PNA monomer building blocks (mainly hydroxyproline derivatives).[25]

2. Results and discussions

According to the procedure outlined in Fig. 2, we have prepared the Boc-PNA[Gly]-OMe (**5a**) monomer, as well as a series of four chiral PNA monomers comprising Ala-, Val-, Leu-, and Phe-derived compounds (Boc-PNA[Ala]-OMe (**5b**), Boc-PNA[Val]-OMe (**5c**), Boc-PNA[Leu]-OMe (**5d**), Boc-PNA[Phe]-OMe (**5e**)).

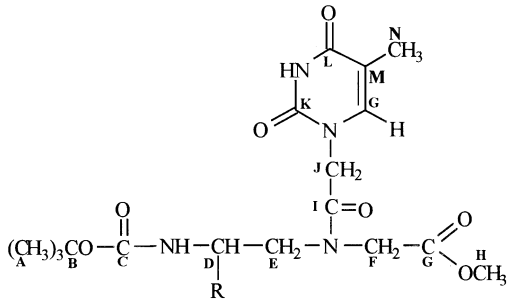
The key step of the synthesis was the Mitsunobu reaction between methyl *N*-(*o*-nitrobenzenesulfonyl)glycinate (*o*NBS-Gly-OMe, **2**), used as an acidic, and *N*-Boc-amino alcohol (Boc-Xaa-ol, **1a**–**e**), used as an alcoholic

Table 1. Yields of PNA monomers and intermediates in their synthesis (%). Yields in references to the table refer to other methods of synthesis

N-, *C*-Protected monomer **5a–e**	Boc-Xaaψ[CH_2N(*o*NBS)]Gly-OMe **3a–e**	Boc-Xaaψ[CH_2NH]Gly-OMe **4a–e**	Boc-PNA[Xaa]-OMe **5a–e**	Total[a] **5a–e**
Boc-PNA[Gly]-OMe	89	65[b]	92	53[c]
Boc-PNA[Ala]-OMe	86	67	88	51
Boc-PNA[Val]-OMe	83	78	75	48[d]
Boc-PNA[Leu]-OMe	78	66	77	40[e]
Boc-PNA[Phe]-OMe	66	81	73	39[f]

[a] Calculated on *o*NBS-Gly-OMe.
[b] Lit.: 83%,[32] 71%.[33]
[c] Lit.: 85%,[32] 43%.[31]
[d] Lit.: 51%.[31]
[e] Lit.: 43%.[9d]
[f] Lit.: 40%.[9d]

Table 2. ^{13}C NMR (400 MHz) chemical shifts of the monomers obtained (major rotamers only)



Monomer	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	R[a]
Boc-PNA[Gly]-OMe	28.727	80.154	156.077	48.054	50.561	49.054	169.835	52.743	167.418	49.054	151.175	164.244	110.891	12.665	140.985	–
Boc-PNA[Ala]-OMe	28.651	80.05	155.508	47.872	52.546	49.069	169.615	52.895	168.115	50.039	151.220	164.282	110.755	12.627	141.219	18.574 (CCH_3)
Boc-PNA[Val]-OMe	28.674	79.50	156.258	47.160	50.842	49.455	169.426	52.562	168.039	49.630	151.228	164.251	110.793	12.612	141.000	17.991 and 18.377 (Val, γC); 30.659 (Val, βC)
Boc-PNA[Leu]-OMe	28.436	79.50	155.612	47.271	52.324	48.703	169.70	52.862	167.279	49.80	150.854	163.855	110.518	12.420	140.747	21.792 and 22.072 (Leu, δC); 41.467 (Leu, βC); 23.367 (Leu, γC)
Boc-PNA[Phe]-OMe	28.613	Unresolved	155.705	47.917	52.009	50.016	169.524	52.584	167.524	49.061	151.129	164.176	110.801	12.642	141.197	39.023 (Phe, βC); 126.999 (Phe, Ph *C*4); 128.628 (Phe, Ph *C*2,6); 129.128 (Phe, Ph *C*3.5); 137.143 (Phe, Ph Cl)

[a] Atom description derived from the skeletons of the respective amino acid

component of the procedure. **2** was obtained from Gly-OMe and *o*NBS-chloride using three procedures with comparably high yields. Boc-Gly-ol **1a** was synthesised from ethanolamine and an excess of $(Boc)_2O$ in dichloromethane, in diethyl ether, or in a *t*-butanol/water system as a solvent, or by a reduction of mixed anhydride obtained from Boc-Gly-OH and isobutyl chloroformate with sodium borohydride.[26] Boc-Phe-ol was synthesised similarly by the reduction of mixed anhydride or by a direct reduction of Boc-Phe-OH using Vitride $[NaAlH_2(OCH_2CH_2OCH_3)_2]$.[27] The latter procedure was also used to obtain Boc-Val-ol with good results. The Mitsunobu reaction was carried out in freshly distilled dry THF, with the use of triphenylphosphine (TPP) and diethyl azodicarboxylate (DEAD) or diisopropyl azodicarboxylate (DIAD), and resulted in fully protected pseudodipeptides with the reduced peptide bond **3a**–**e**. The pseudodipeptides were obtained in high yields after purification by flash chromatography (Table 1). The choice of the solvent system for chromatographic separation of the reaction products was in some cases a problematic issue, due to the similarity of chromatographic behaviour of dialkyl hydrazine dicarboxylates (invisible under UV light, distinctly developed with a cerium–molybdate reagent) and the desired products.

The *o*NBS group was removed by thiolysis. The thiolysis conditions were optimised using *o*NBS-Gly-OMe or Boc-Glyψ[CH_2N(*o*NBS)]Gly-OMe as model compounds, with all possible combinations of three tiols (2-mercaptoethanol, 1,2-ethanedithiol, tiophenol) and four bases (potassium carbonate, diisopropylethylamine, piperidine, and DBU [imidazole-1,8-diazabicyclo[5.4.0]undec-7-ene]). The use of a three-fold excess of thiol and a 50% excess of base in DMF or acetonitrile resulted in a high conversion ratio observed using HPLC. The procedure employing thiophenol and potassium carbonate in acetonitrile was selected as the most convenient for isolation of partially protected products. The pseudopeptides were isolated from the reaction mixture using a diethyl ether/water work-up to obtain **4a**–**e** in rather high purity (TLC inspection) and satisfactory yields (Table 1). Our earlier attempts[9g] to use standard C_{18} cartridges or liquid chromatography on silica gel for the isolation and purification of the pseudopeptides gave worse results, as far as yield and purity is concerned.

Thymin-l-ylacetic acid (ThyAcOH) was synthesised from thymine and ethyl bromoacetate analogously to the procedure described[28] and was subsequently coupled to the peptidic part of PNA monomers using an optimised procedure[29] employing EDC and HODhbt. Fully protected monomers **5a**–**e** were purified by RP-HPLC chromatography and obtained in total 40–53% yields. The products may also be effectively purified by low pressure liquid chromatography (not reported here).

The structure of the final products was confirmed by analysis of ^{1}H- and ^{13}C NMR (Table 2), IR and MS spectra. The ^{1}H- and ^{13}C NMR spectra indicate the presence of two rotamers around the tertiary amide bond. Temperature dependence of the rotamer properties has been next investigated using high-resolution NMR.[9i]

The fully-protected monomers obtained are ready to use in both solution and solid-phase PNA oligomer synthesis after deprotection of the C-terminal carboxylic group using one of the standard methyl ester cleavage procedures.

3. Conclusions

In summary, we have developed a new and convenient method of PNA monomer synthesis. Since the deprotection of the secondary amine group occurs under mild conditions, the procedure seems to be of general applicability and allows various modifications of the PNA structure. The described procedure can be easily used in the synthesis of PNA monomers containing chiral amino acid residues not only in the 'aminoethyl', but also in the 'amino acid' part of the PNA backbone [for a review on PNA modifications see Ref. 2], by using various protected β-amino alcohols and α-amino acid esters. It should be noted that a similar procedure may also be effective in solid-phase synthesis of PNA monomers, using the Mitsunobu reaction.[30] In addition, our results suggest that the proposed method gives products of higher optical activity in comparison with those obtained using the reductive amination of *N*-protected α-amino aldehydes with amino esters.[9c,31]

4. Experimental

4.1. General

All NMR spectra were recorded in $CDCl_3$. The 100 MHz ^{1}H NMR spectra were recorded on a BF-567A spectrometer (Tesla, Brno), the 400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were recorded on a Varian Unity spectrometer using tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported in ppm downfield TMS. For analytical RP-HPLC, a Varian Vista 5500 system and a Kromasil 5 column (4.6×250 mm, C18100-5C8), for semi-preparative HPLC, a Kromasil column (25×250 mm, C8, 5 μm) were used, eluent monitored at λ=254 and/or 223 nm. Gradient elutions composed of A (0.1% TFA in water) and B (0.08% TFA in 80% acetonitrile/20% water) were used. Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} sheets, using the following solvent systems (by volume): A (ethyl acetate/petroleum ether 1:1), B (chloroform/methanol 9:1), and C (ethyl acetate); compounds were located on plates using UV observation when appropriate, then developed with a ninhydrine reagent (at 150°C) and next with a cerium–molybdate reagent (at 150°C). For flash column chromatography silica gel 60 was used. Melting points were uncorrected. Optical rotations were measured at 19–22°C with a Jansen JD-20 spectropolarimeter (results ±0.5°), IR spectra with a Bruker IFS 66 spectrometer and reported as ν_{max}/cm^{-1}. MS (FAB) spectra were recorded with a TRIOS quadrupole mass spectrometer (MASSLAP) at Gdańsk University, HRMS spectra with a AMD 604 mass spectrometer at Poznań University, Poland, and reported as *m*/*z*. Evaporations were performed under diminished pressure at <50°C. Commercially available reagents: acetonitrile, methanol, diethyl ether, toluene, inorganic chemicals were used. Dichloromethane, diisopropylethylamine, ethyl acetate and petroleum ether were distilled prior to use; the

used petroleum ether refers to the bp 50–65°C fraction. Tetrahydrofuran p.p.a. freshly distilled over natrium hydride was used for the Mitsunobu procedure. Boc-Ala-ol (**1b**) and Boc-Leu-ol (**1d**) were purchased from a commercial source (Chem-Impex Int. Inc., Wood Dale, IL, USA). Boc-Gly-ol (**1a**) was obtained using either (a) ethanolamine, Boc_2O, and TEA i dichloromethane (colourless oil, 70% yield), (b) ethanolamine and Boc_2O in diethyl ether (42% with minor impurities), (c) ethanolamine, Boc_2O, and NaOH in a mixture of *t*-butanol and water (48% yield, with minor impurities), (d) the procedure of Rodriguez et al.[26] with THF as a solvent (58% yield).

4.1.1. Methyl *N*-(*o*-nitrophenylsulfonyl)-glycinate (*o*NBS-Gly-OMe) 2. To a vigorously stirred, ice-cooled suspension of methyl glycinate hydrochloride (obtained using standard procedure, 10.40 g, 80 mmol) and TEA (23.50 mL, 168 mmol) in dichloromethane (120 mL), *o*-nitrophenylsulfonyl chloride 95% (Fluka, 20.50 g, 88 mmol) was added portion-wise during 30 min. The mixture was allowed to warm to room temperature and it was stirred overnight (although the reaction was almost complete after 1 h, according to TLC and HPLC analyses, the conversion rate by HPLC was ca. 100%). The solvent from the resulting brown-coloured solution was evaporated and the mixture was partitioned between ethyl acetate (150 mL) and water (100 mL). The water layer was washed with ethyl acetate (50 mL) and the combined organic layers were subsequently washed with water (1×25 mL), 1 M HCl (2×25 mL), brine (2×25 mL), 5% $NaHCO_3$ (2×25 mL) and brine (2×25 mL). The organic layer was dried over anhydrous $MgSO_4$, reduced under vacuum to ca. 100 mL and petroleum ether (ca. 20 mL) was added. On the next day **2** (20.56 g, yield 94%) was obtained as pale yellow crystals (homogenous in TLC). When THF was used as a reaction solvent, the conversion rate by HPLC was 95% and the yield of the isolated product 88%; similarly, when a mixture of DCM/DMF (4:1, v/v) was used as a solvent and DIEA as a base, the conversion rate by HPLC was 99%, the yield of the isolated product 87%. Mp: 108–109°C. TLC: R_F=0.22 (A). HPLC: R_T= 20 min (0:100%A, 0%B; 30:0%A, 100%B). IR (film): 618m; 655m; 707m; 730m; 740s; 785s; 838m (C–N); 855m; 879w; 983m; 1006w; 1059w; 1127s (S–O); 1164s (C–O, S–O), 1226s 1261m; 1300m; 1347s (N–O, S–O); 1369s (C–H); 1402s; 1445m; 1540s (N–O); 1595m; 1707m; 1743s (C═O), 2957w; 2985w; 3099w; 3341s (N–H). ^{1}H NMR (100 MHz): δ=3.64 (3H, s, OCH_3); 4.05 (2H, d, NCH_2CO, J=5.5 Hz); 6.16 (IH, t, N*H*, J=5.5 Hz), 7.67–8.35 (4H, m, Ar*H*).

4.1.2. Boc-Val-ol 1c.[34] To a stirred, ice-cooled 70% w/w solution of sodium bis(2-methoxyethoxy)aluminium hydride (Vitride) in toluene (Lancaster, 7.2 mL, 15 mmol), a solution of Boc-Val-OH (1.77 g, 8.15 mmol) in dry THF (10 mL) was added dropwise over 2 h, the resulting solution obtained was stirred for an additional 2 h at 0°C. MeOH (5 mL) was added dropwise (CAUTION! a strong evolution of gas was observed), followed by 100 mL of saturated potassium sodium tartarate. The mixture was stirred for an additional 2 h at room temperature, the water layer was extracted by ethyl acetate (3×100 mL); the collected organic layers were washed with water (3×30 mL), 5% $NaHCO_3$ (3×30 mL), and brine (3×30 mL), dried over anhydrous $MgSO_4$ and evaporated, yielding 1.19 g (5.87 mmol, 72% yield) of homogenous (TLC) oily **1c** (lit.: oil[35a]). TLC: R_F=0.60 (A). $[\alpha]_{589\ nm}^{rt}$=−15.5 (c=1, MeOH). IR (film): 757m; 863w; 900w; 923w; 981m; 1028m; 1049m (C–O); 1078m (C–O); 1118w; 1173s; 1249m (*t*-butyl); 1311m; 1367s, 1392m (*t*-butyl); 1468m; 1514s (N–H); 1693s (C(O)N); 2876m; 2934m; 2966s (C–H); 3351s (N–H; O–H). ^{1}H NMR (100 MHz): δ= 0.80–1.06 (6H, dd, CH(CH_3)$_2$); 1.49 (9H, s, C(CH_3)$_3$); 1.88 (1H, m, C*H*(CH$_3$)$_2$); 3.50 (1H, q, NC*H*CO); 3.76 (3H, m, CH_2O*H*); 4.45 (1H, br, CON*H*).

4.1.3. Boc-Phe-ol 1e. *Procedure A*. Analogously to the synthesis of **1c**; starting from Boc-Phe-OH (1.32 g, 5 mmol), 0.99 g of **1e** was obtained as colourless oil (yield 79%; lit.: crystals, mp: 90–91°C,[36] 93–94°C,[37] 93–95°C,[26] 94.5°C,[38] 94–96°C[35b]). TLC: R_F=0.65 (A). HPLC: R_T=27 min (0: 100% A, 0% B; 30: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−25.0 (c=1, MeOH) (lit.: −26, c=1.05, MeOH[26]). IR (film): 660m; 700m (Ar–H); 738m; 753m (Ar–H); 776w; 850w; 886w; 1006s (C–O); 1035m; 1052m; 1086w; 1171s; 1251m, 1268m (*t*-butyl); 1316m; 1366m, 1390m (*t*-butyl); 1445 (C═C); 1497w (C═C); 1528s (N–H); 1602w (C═C); 1688s (C(O)N); 2874m; 2933m; 2981m (C–H); 3006w; 3025w, 3062w (Ar–H); 3358s (N–H; O–H).

Procedure B. The procedure of Rodriguez et al.[26] was used with THF as a solvent. Yield 79%, crystals, mp 91–92°C.

4.2. Synthesis of the protected pseudodipeptide 3a–e methyl esters (general procedure)

*o*NBS-Gly-OMe, **2** (0.55 g, 2 mmol), Boc-Xaa-ol, **1a–e** (3 mmol), and triphenylphosphine, Ph_3P (0.79 g, 3 mmol), were dissolved in 20 mL of freshly distilled dry THF. The solution was stirred in an ice bath under nitrogen, and diethyl azodicarboxylate, DEAD (0.47 mL, 3 mmol), or diisopropyl azodicarboxylate, DIAD (0.59 mL, 3 mmol), was added in 3 portions over 10 min. The reaction was monitored by HPLC. After overnight stirring under nitrogen, the solvent was evaporated and the oily residue was residue was redissolved in 30 mL of Et_2O and cooled to 0°C. On the other day the white precipitate (a mixture of TPP oxide and dialkyl hydrazinedicarboxylate) was filtered off and the solvent was evaporated. The residue was subjected to separation on silica gel in an ethyl acetate/petroleum ether system (1:6–1:3, v/v), yielding chromatographically homogeneous protected pseudodipeptide methyl esters **3a–e**.

4.2.1. Boc-Glyψ[CH$_2$N(*o*NBS]Gly-OMe 3a. 1.10 g of **2** as used, 1.49 g of **3a** was obtained as a pale yellow oil (yield 89%). TLC: R_F=0.44 (A). HPLC: R_T=24 min (0: 100% A, 0% B; 30: 0% A, 100% B). IR (film): 652w; 690w; 740m; 774m; 852m (C–N); 904m; 939m; 963m; 996m; 1060m; 1131m; 1166s (C–O, S–O); 1220m; 1248m (*t*-butyl); 1275m; 1367m (N–O, S–O); 1392m (*t*-butyl); 1440m (C═C); 1513m (N–H); 1546s (N–O); 1590w (C═C); 1708s (C(O)N); 1752s (CC(O)OC); 2979m (C–H); 3096w (Ar–H); 3425m (N–H). ^{1}H NMR (100 MHz): δ=1.49 (9H, s, C(CH_3)$_3$); 3.37 (2H, t, C(O)NCH_2CH_2N, J=5.5 Hz); 3.55 (2H, m, C(O)NCH_2CH$_2$N, J=5.5 Hz); 3.75 (3H, s, CH_3;

4.27 (2H, s, NCH_2CO); 5.15 (1H, br, N*H*); 7.63–8.00 (3H, m, Ar*H*4-6); 8.15 (1H, m, Ar*H*3).

4.2.2. Boc-Alaψ[CH$_2$N(*o*NBS]Gly-OMe 3b. 0.55 g of **2** was used, 0.74 g of **3b** was obtained as a yellow oil (yield 86%). TLC: R_F=0.50 (A). HPLC: R_T=28 min (0: 100% A, 0% B; 30: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−15.5 (*c*=1, MeOH). MS (FAB), calculated (found): (M+H$^+$)=432 (432, 332 [M+2H-Boc]$^+$). IR (film): 652w; 698w; 741m; 780s; 852m (C–N); 903m; 934m; 952m; 993w; 1061s; 1098w; 1126m; 1165s (C–O, S–O); 1221s; 1369s (N–O, S–O); 1455m (C=C); 1516m (N–H); 1546s (N–O); 1590w (C=C); 1705s (C(O)N); 1752s (CC(O)OC); 2979m (C–H); 3097w (Ar–H); 3367m (N–H). ^{1}H NMR (400 MHz): δ= 1.144mi[†], 1.168ma[‡] (3H, 2d, CHCH_3, *J*=7.2 Hz); 1.419ma, 1.446mi (9H, 2s, C(CH_3)$_3$); 3.348mi, 3.384ma (2H, 2d, NCHCH_2N, *J*=4.8 Hz); 3.497 (1H, m, NC*H*CH$_2$N); 3.609mi, 3.662ma (3H, 2s, OCH_3); 4.178–4.231 (2H, m, NCH_2CO); 4.647 (1H, br, N*H*); 7.610 (1H, m, Ar*H*5); 7.60–7.755 (2H, m, Ar*H*4,6); 8.031 (1H, m, Ar*H*3).

4.2.3. Boc-Valψ[CH$_2$N(*o*NBS]Gly-OMe 3c. 0.55 g of **2** was used, 0.76 g of **3c** was obtained as a pale yellow oil (yield 83%). TLC: R_F=0.66 (A). HPLC: R_T=30 min (0: 100% A, 0% B; 30: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−13 (*c*=1, MeOH). IR (film): 652w; 740m; 778s; 852m (C–N); 935m; 1063s; 1167s (S–O, C–O); 1226s; 1369s (N–O, S–O); 1546s (N–O); 1714s (C(O)N); 1745s (CC(O)OC); 2981m (C–H); 3303m (N–H). ^{1}H NMR (100 MHz): δ=0.93 (3H, d, CH(CH$_3$)CH_3, *J*=7.0 Hz); 0.95 (3H, d, CH(CH$_3$)CH_3, *J*=7.0 Hz); 1.43ma, 1.48mi (9H, 2s, C(CH_3)$_3$); 1.72 (1H, m,C*H*(CH$_3$)$_2$); 3.45–3.90 (3H, m, NC*H*CH_2N); 3.68 (3H, s, OCH_3); 4.37 (2H, AB, NCH_2CO, *J*=20.0 Hz); 4.75 (1H, br d, N*H*, *J*≈8.0 Hz); 7.67–7.92 (3H, m, Ar*H*4-6); 8.11 (1H, m, Ar*H*3).

4.2.4. Boc-Leuψ[CH$_2$N(*o*NBS)]Gly-OMe 3d. 0.82 g of **2** was used, 1.11 g of **3d** was obtained as a colourless crystals after additional crystallization from petroleum ether/ethyl acetate system (yield 78%). Mp: 114–115°C. TLC: R_F= 0.69 (A). HPLC: R_T=31 min (0; 100% A, 0% B; 30; 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−30 (*c*=1, MeOH). IR (KBr): 464w; 527m; 546m; 570m; 586s; 651m; 698m; 729m; 739m; 770m; 781m; 826m; 851m; 893w; 926m; 936m; 956w; 993w; 1025m; 1043w; 1081m; 1092m; 1126m; 1171s (S–O, C–O); 1223m; 1252m; 1286m; 1352m; 1365s (N–O, S–O); 1418w; 1439m; 1453m; 1542s (N–O); 1588w; 1665s; 1704s (C(O)N); 1753s (CC(O)OC); 2872m; 2961s (C–H); 3347s (N–H). ^{1}H NMR(100 MHz): δ=0.90 (6H, d, CH(CH_3)$_2$, *J*=6.5 Hz); 1.30 (2H, m, CH_2CH(CH$_3$)$_2$); 1.45 (9H, s, C(CH_3)$_3$); 1.71 (1H, m, C*H*(CH$_3$)$_2$); 3.40–3.63 (3H, m, NC*H*CH_2N); 3.68 (3H, s, OCH_3); 4.35 (2H, AB, NCH_2CO, *J*=20.0 Hz); 4.65 (1H, br d, N*H*, *J*≈10.0 Hz); 7.67–7.95 (3H, m, Ar*H*4-6); 8.12 (1H, m, Ar*H*3).

4.2.5. Boc-Pheψ[CH$_2$N(*o*NBS)]Gly-OMe 3e. 1.10 g of **2** was used, 1.34 g of **3e** was obtained as a pale yellow oil (yield 66%). TLC: R_F=0.71 (A). HPLC: R_T=31 min (0: 100% A, 0% B; 30: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−15.5 (*c*=1, MeOH). IR (film): 652m; 701m; 740m; 757m; 777m; 825w; 852m; 894w; 940m; 1025m; 1048m; 1127m; 1164s (S–O, C–O); 1216m; 1247m; 1367s (N–O, S–O); 1439m; 1454m; 1512m; 1545s (N–O); 1590w; 1705s (C(O)N); 1752s (CC(O)OC); 2978m (C–H);3397m (N–H). ^{1}H NMR (100 MHz): δ=1.39 (9H, s, C(CH_3)$_3$); 2.85 (2H, d, CH_2Ph, *J*=6.5 Hz); 3.45–3.78 (3H, m, NC*H*CH_2N); 3.65 (3H, s, OCH_3); 4.02 (1H, m, C*H*CH$_2$Ph); 4.20 (2H, AB, NCH_2CO, *J*=20.0 Hz); 4.75 (1H, d, N*H*, *J*=8.5 Hz); 7.17–7.50 (5H, m, Ph*H*2-6); 7.65–7.90 (3H, m, *o*NBS Ar*H*4-6); 8.02 (1H, m, *o*NBS Ar*H*3).

4.3. Removal of the *o*NBS group by thiolysis (4a–e, general procedure)

To the protected pseudodipeptide **3a**–**e** (1.6 mmol) dissolved in 25 mL of acetonitrile, potassium carbonate (0.33 g, 2.4 mmol) and thiophenol (0.33 mL, 3.2 mmol) were added; the resulting solution was vigorously stirred overnight, the solvent was evaporated and the crude product was partitioned between diethyl ether (30 mL) and 10% aqueous citric acid (100 mL). The aqueous layer was washed with diethyl ether (2×20 mL) and the combined ether layers were extracted with 10% aqueous citric acid (1×20 mL). To the collected water layers solid potassium carbonate was added in a high excess (CAUTION! a strong evolution of gas was observed). The water layers were extracted with diethyl ether (4×50 mL), the resulting ether layers were washed with brine (2×20 mL) and dried over anhydrous $MgSO_4$. In the pseudopeptide products **4a**–**e** obtained after evaporation, only minor impurities could be detected (TLC inspection); the products were used in subsequent procedures without additional purification.

4.3.1. Boc-Glyψ(CH$_2$HN)Gly-OMe 4a. 1.35 g (3.24 mmol) of **3a** was used, 0.49 g (2.10 mmol, 65% yield) of a colourless homogenous oily product was obtained (lit.: oil).[32,33] TLC: R_F=0.13 (B), R_F=0.23 (C). HPLC: R_T= 27 min (0: 100% A, 0% B; 60: 0% A, 100% B). IR (film): 608m; 699w; 780m; 866m (N–H); 947m; 997m; 1050m; 1168s (O–C); 1249m (*t*-butyl); 1275m; 1365m, 1392m (*t*-butyl); 1454m (–CH$_2$–); 1514s (N–H); 1713s (C(O)N); 1743m (C(O)O); 2933m; 2975s, 3352s (N–H).

4.3.2. Boc-Alaψ(CH$_2$NH)Gly-OMe 4b. 0.47 g (1.09 mmol) of **3b** was used, 0.18 g (0.73 mmol, 67% yield) of a colourless homogeneous oily product was obtained. TLC: R_F=0.15 (A), R_F=0.42 (C). $[\alpha]_{589nm}^{rt}$=−4 (*c*=1, MeOH). IR (film): 780m; 849m; 882w; 1067m; 1172s (C–O); 1246m (*t*-butyl); 1367s, 1391m (*t*-butyl); 1455m; 1522s; 1709s (C(O)N); 1743s (CC(O)OC); 2931m; 2977s (C–H); 3340s (N–H). ^{1}H NMR (400 MHz): δ=1.146 (3H, d, CHCH_3, *J*=6.8 Hz); 1.444 (9H, s, C(CH_3)$_3$); 1.982 (1H, br, CH$_2$N*H*CH$_2$); 2.628 (2H, d, CHCH_2NH, *J*=6.0 Hz); 3.423 (2H, AB, NCH_2CO, *J*=17.6 Hz); 3.717 (1H, m, NHC*H*CH$_2$); 3.727 (3H, s, OCH_3); 4.818 (1H, br, CON*H*).

4.3.3. Boc-Valψ(CH$_2$NH)Gly-OMe 4c. 0.75 g (1.63 mmol) of **3c** was used, 0.35 g (1.27 mmol, 78% yield) of a colorless homogenous oily product was obtained. TLC: R_F=0.15 (A), 0.49 (B), 0.59 (C). $[\alpha]^{rt}_{589\ nm}$=−3 (*c*=1, MeOH). IR (film): 701m; 744m; 851w; 877w; 1028m; 1048m; 1168s (C–O); 1205m, 1246m (*t*-butyl); 1366s,

[†] mi—minor
[‡] ma—major

1391m (*t*-butyl); 1454m; 1496s; 1606w; 1708s (C(O)N); 1742s (CC(O)OC); 2854m; 2925s (C–H); 3027; 3062; 3343s (N–H). ^{1}H NMR (400 MHz): δ=0.874 (3H, d, CH(CH$_3$)C*H*$_3$, *J*=6.8 Hz); 0.899 (3H, d, CH(C*H*$_3$)CH$_3$, *J*=6.8 Hz); 1.416 (9H, s, C(C*H*$_3$)$_3$); 1.748 (1H, m, C*H*(CH$_3$)$_2$); 2.021 (1H, br, CH$_2$N*H*CH$_2$); 2.624 (2H, d, CHC*H*$_2$NH, *J*=5.2 Hz); 3.390 (2H, AB, NC*H*$_2$CO, *J*=17.6 Hz); 3.464 (1H, m, NHC*H*CH$_2$); 3.694 (3H, s, OC*H*$_3$); 4.725 (1H, br, CON*H*).

4.3.4. Boc-Leuψ(CH$_2$NH)Gly-OMe 4d. 1.05 g (2.22 mmol) of **3d** was used, 0.42 g (1.46 mmol, 66% yield) of a colourless homogenous oily product was obtained. TLC: R_F=0.57 (C). $[\alpha]_{589\ nm}^{rt}$=−14.5 (*c*=1, MeOH) IR (film): 1173s (C–O); 779m; 846w, 870w; 898w; 937w; 1022 m; 1044m; 1174s; 1228s (*t*-butyl); 1366s, 1390m (*t*-butyl); 1438m; 1447m; 1469m; 1524s; 1605w; 1707s (C(O)N); 1744s (CC(O)OC); 2870m, 2880m, 2956s, 2996s (C–H); 3345s (N–H). ^{1}H NMR (400 MHz): δ=0.921 (3H, d, CH(CH$_3$)C*H*$_3$); *J*=6.6 Hz); 0.923 (3H, d, CH(C*H*$_3$)CH$_3$), *J*=6.6 Hz); 1.24–1.39 (2H, m, C*H*$_2$CH(CH$_3$)$_2$); 1.443 (9H, s, C(C*H*$_3$)$_3$); 1.662 (1H, m, C*H*(CH$_3$)$_2$); 1.977 (1H, br, CH$_2$N*H*CH$_2$); 2.642 (2H, d, CHC*H*$_2$NH, *J*=5.2 Hz); 3.431 (2H, AB, NC*H*$_2$CO, *J*=17.2 Hz); 3.724 (3H, s, OC*H*$_3$); 3.74 (1H, br, NHC*H*CH$_2$); 4.655 (1H, br, CON*H*).

4.3.5. Boc-Pheψ(CH$_2$NH)Gly-OMe 4e. 0.57 g (1.12 mmol) of **3e** was used, 0.29 g (0.91 mmol, 81% yield) of a colourless homogenous oily product was obtained TLC: R_F=0.15 (A). $[\alpha]_{589\ nm}^{rt}$=−5.5 (*c*=1, MeOH). IR (film): 702s (Ar–H); 746m (Ar–H); 852w; 877w; 946w; 1029m; 1048m; 1171s (C–O); 1207m, 1247m (*t*-butyl); 1366s, 1391m (*t*-butyl); 1438m; 1455m; 1497s; 1603w; 1710s (C(O)N); 1742s (CC(O)OC); 2857m, 2930m, 2953m, 2976m (C–H); 3027m, 3062w (Ar–H); 3347s (N–H). ^{1}H NMR (400 MHz): δ=1.402 (9H, s, C(C*H*$_3$)$_3$); 2.100 (1H, br, CH$_2$N*H*CH$_2$); 2.615 (2H, d, CHC*H*$_2$NH, *J*=5.6 Hz); 2.754 (1H, dd, CHC*H*$_2$Ph, $J_{\alpha\beta}$=7.2 Hz, $J_{\beta\beta}$=13.6 Hz); 2.843 (1H, br, CHC*H*$_2$Ph); 3.367 (2H, AB, NC*H*$_2$CO, *J*=17.4 Hz); 3.682 (3H, s, OC*H*$_3$); 3.880 (1H, br, C*H*CH$_2$Ph); 5.005 (1H, d, CON*H*, *J*=8.4 Hz); 7.162–7.295 (5H, m, Ph*H*).

4.4. Acylation of the pseudodipeptides with thymin-1-ylacetic acid (5a-e, general procedure)

Thymin-1-ylacetic acid (2.02 g, 11 mmol), HODhbt (1.78 g, 11 mmol), DIEA (1.92 mL, 11 mmol) and pseudopeptide **5a**–**e** (10 mmol) were suspended in 25 mL of vigorously stirred dry dimethylformamide and cooled to 0°C. Then 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC hydrochloride, 2.10 g, 11 mmol) was added portionwise during 30 min at 0°C. The mixture was allowed to warm to room temperature pH was kept at 8–9 by DIEA addition. After 24–36 h the solvent was evaporated and the residue was partitioned between water (50 mL) and ethyl acetate (150 mL). The organic phase was washed with 0.1 M $KHSO_4$ (2×50 mL), water (4×50 mL), brine (1×50 mL), and dried over anhydrous $MgSO_4$. The solvent was evaporated to dryness. The crude product was dissolved in a 15–25% solution of acetonitrile in water and purified on a preparative HPLC column using a linear gradient elution of 0–60 or 20–80% acetonitrile in water over 90 min; eluent was monitored at 254 nm. Fractions containing >95% of the desired product were pooled and lyophilized.

4.4.1. Boc-Glyψ[CH$_2$N(ThyAc)]Gly-OMe (Boc-PNA-[Gly]-OMe) 5a. 0.45 g (1.94 mmol) of **4a** was used, 1.22 g of a crude product was obtained as a pale yellow oil. 0.212 g of the crude material was purified using RP-HPLC, 0.123 g (0.310 mmol) of a chromatographically homogenous lyophilizate was obtained (92% yield) (lit.: white crystals, mp: 157°C).[32] TLC: R_F=0.44 (B), 0.23 (C). HPLC: R_T=19 min (0: 100% A, 0% B; 30: 0% A, 100% B). MS (FAB), calculated (found): (M+H$^+$)=399.2 (399.1, 299.3 [M+2H-Boc]$^+$). HRMS, calcd (found): (M+H$^+$)=399.18799 (399.18806). IR (film): 666w; 756s; 852w; 892w; 965w; 998w; 1146w; 1173s (C–O); 1217s (*t*-butyl); 1318m; 1368s, 1388m (*t*-butyl); 1409m; 1448m; 1523s; 1580w; 1605m; 1682s (OC(O)N, CC(O)N); 1745s (CC(O)OC); 2853w; 2882, 2896m (C–H), 3106m (Ar–H); 3328s (N–H).

4.4.2. Boc-Alaψ[CH$_2$N(ThyAc)]Gly-OMe (Boc-PNA-[Ala]-OMe) 5b. 0.18 g (0.75 mmol) of **4b** was used, 0.40 g of a crude product was obtained as a pale yellow oil. 0.200 g of the crude material was purified using RP-HPLC, 0.136 g (0.658 mmol) of a chromatographically homogenous lyophilizate was obtained (88% yield). TLC: R_F=0.36 (B), 0.26 (C). HPLC: R_T=5.6 min (isocratic elution 56% A, 44% B), 19 min (0: 100% A, 0% B; 30: 0% A, 100% B), 30 min (0: 100% A, 0% B; 60: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−4.5 (*c*=1, MeOH). MS (FAB), calcd (found): (M+H$^+$)=413.2 (413.3, 313.2 [M+2H-Boc]$^+$). HRMS, calcd (found): (M+H$^+$)=413.20361 (413.20315). IR (film): 470m; 537m; 551m; 595m; 642w; 683w; 733w; 849w; 867w; 908m; 942w; 989s; 1053s; 1067s; 1124m; 1175s (C–O); 1215s, 1250s (*t*-butyl); 1315m; 1350m; 1366s, 1380m (*t*-butyl); 1387m; 1413m; 1438m; 1474s; 1524s; 1540w; 1584w; 1677s, 1691s (OC(O)N, CC(O)N); 1750s (CC(O)OC); 2850m; 2916s; 2977s (C–H); 3180s (Ar–H); 3332s (N–H). ^{1}H NMR (400 MHz): δ=1.132mi, 1.256ma (3H, 2d, CHC*H*$_3$, *J*=6.4 Hz); 1.411ma, 1.432mi (9H, 2s, C(C*H*$_3$)$_3$); 1.904ma, 1.920mi (3H, 2s, Thy-C*H*$_3$); 3.360ma, 3.528mi (2H, 2m, CHC*H*$_2$NH); 3.730ma, 3.806mi (3H, 2s, OC*H*$_3$); 3.897 (1H, m, NHC*H*CH$_2$); 4.240 (2H, AB, NC*H*$_2$COOCH$_3$, *J*=22.4 Hz); 4.456 (2H, AB, NC*H*$_2$CON, *J*=16.0 Hz); 5.155 (1H, br, CON*H*); 7.042mi, 7.080ma (1H, 2s, Thy-*H*); 9.277mi, 9.360ma (1H, 2 br s, Thy-N*H*);

4.4.3. Boc-Valψ[CH$_2$N(ThyAc)]Gly-OMe (Boc-PNA-[Val]-OMe) 5c. 0.30 g (1.09 mmol) of **4c** was used, 0.86 g of a crude product was obtained as a pale yellow oil. 0.198 g of the crude material was purified using RP-HPLC, 0.083 g (0.189 mmol) of a chromatographically homogenous lyophilizate was obtained (75% yield). TLC: R_F=0.39 (B), 0.44 (C). HPLC: R_T=7 min (isocratic elution 50% A, 50% B), 22 min (0: 100% A, 0% B; 30: 0% A, 100% B), 33 min (0: 100% A, 0% B; 60: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=+16.5 (*c*=1, MeOH) [lit.[31] +8 (*c*=1, MeOH)]. MS (FAB), calcd (found): (M+H$^+$)=441.2 (441.2, 341.2 [M+2H-Boc]$^+$). HRMS, calcd (found): (M+H$^+$)=441.23492 (441.23475). IR (film): 665m; 755s; 825m (N–H); 896w; 941w; 965w; 1016w; 1065w; 1174m (O–C); 1213m, 1240m (*t*-butyl); 1367m, 1389m; 1415m; 1470m; 1519s (N–H); 1673s, 1693s (OC(O)N, CC(O)N); 1749m (C(O)O); 2128w; 2965s (C–H); 3193m; 3334m; (N–H).

4.4.4. Boc-Leuψ[CH_2N(ThyAc)]Gly-OMe (Boc-PNA-[Leu]-OMe) 5d. 0.71 g (2.44 mmol) of **4d** was used, 1.89 g of a crude product was obtained as a yellow oil. 0.199 g of the crude material was purified using RP-HPLC, 0.089 g (0.198 mmol) of a chromatographically homogenous lyophilizate was obtained (77% yield). TLC: R_F= 0.39 (B), 0.50 (C). HPLC: R_T=10.6 min (isocratic elution 50% A, 50% B), 24 min (0: 100% A, 0% B; 30: 0% A, 100% B), 39 min (0: 100% A, 0% B; 60: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=−5.5 (c=1, MeOH) [lit.[9d] −2.1 (c=1, MeOH)]. MS (FAB), calcd (found): ($M+H^+$)=455.3 (455.3, 355.2 [M+2H-Boc]$^+$). HRMS, calculated (found): ($M+H^+$)=455.25058 (455.25033). IR (film): 411m; 463m; 554w; 665w; 716w; 756m; 812w; 838w; 896w; 939w; 964w; 1014m; 1049m; 1067m; 1121w; 1168s (C–O); 1210s (*t*-butyl); 1247m; 1319w, 1367s (*t*-butyl); 1388m; 1405w, 1470s; 1520m; 1551w; 1559w; 1668s, 1692, 1714s (OC(O)N, CC(O)N); 1752s (CC(O)OC); 2872m; 2933m; 2958s (C–H); 3007m; 3063m; 3122m (Ar–H); 3208m; 3338s (N–H).

4.4.5. Boc-Pheψ[CH_2N(ThyAc)]Gly-OMe (Boc-PNA-[Phe]-OMe) 5e. 0.194 g (0.60 mmol) of **4e** was used, 0.42 g of a crude product was obtained as a yellow oil. 0.249 g of the crude material was purified using RP-HPLC, 0.127 g (0.260 mmol) of a chromatographically homogenous lyophilizate was obtained (73% yield). TLC: R_F=0.54 (B), 0.45 (C). HPLC: R_T=5.5 min (isocratic elution 37% A, 63% B), 24 min (0: 100% A, 0% B; 30: 0% A, 100% B), 40 min (0: 100% A, 0% B; 60: 0% A, 100% B). $[\alpha]_{589\ nm}^{rt}$=+6.5 (c=1, MeOH) [lit.[9d] +6.7 (c=1, MeOH)]. MS (FAB), calcd (found): ($M+H^+$)= 489.2 (489.3, 389.1 [M+2H-Boc]$^+$). HRMS, calcd (found): ($M-H^+$)=489.23492 (489.23574). IR (film): 404m; 460m; 478m; 534w; 652w; 702m; 756m; 810m; 834w; 856m; 920w; 999m; 1026m; 1045m; 1096m; 1121w; 1172s (C–O); 1199m; 1210s; 1228s (*t*-butyl); 1244m; 1278m; 1299m; 1335w, 1360s (*t*-butyl); 1374m; 1394m; 1417w, 1470s; 1490m; 1533s; 1551w; 1559w; 1660s, 1686s, 1724s (OC(O)N, CC(O)N); 1747s (CC(O)OC); 2872m; 2934m; 2949m; 2975m; 2990m; 3058w; 3112w; 3248m; 3344s (N–H).

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