88. Synthesis, Enzymic Degradation, Lipophilic Properties, and Biological Activity of [D-Alanine[2], *t*-butylglycine[5]]enkephalin Amide[1])

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Summary

L-C^{α}-*t*-Butylglycine (Bug), its amide, methyl ester, and N^{a}-*t*-butoxycarbonyl derivative were prepared by an asymmetric synthesis, and the *Hansch* side-chain hydrophobic (lipophilicity) parameter determined. A new enkephalin analogue, H · Tyr-D-Ala-Gly-Phe-Bug · NH_2 was synthesized which is pharmacologically active in two *in vitro* assays and strongly resistant against a number of enzymes *in vitro*.

Introduction. – We have recently become interested in the use of so-called 'fat' or 'super' amino acids for the study of structure-activity relationships in the field of neuroactive peptides, including the morphinomimetic enkephalins [1]. Fat amino acids exhibit enhanced lipophilicity (fat in the sense of fatty), altered steric requirements (fat in the sense of bulky), different electronic properties (*e.g.* degrees of aromaticity), and resistance against enzymic degradation with respect to the corresponding proteinogenic amino acids.

So far we have prepared peptides containing L-carboranylalanine [2], L-adamantylalanine [3], and L-neopentylglycine (γ-methylleucine[2]). Some of these peptides had remarkable properties compared to those with the natural amino-acid sequences, *e.g.* enhanced receptor binding [4–6], increased pharmacological potency [1] [7], and greater resistance towards a protease [4].

A further interesting fat amino acid is L-C^{α}-*t*-butylglycine (*t*-butylglycine, Bug, *t*-leucine, or β-methylvaline). This fat valine was described many years ago by *Knoop & Landmann* [8], *Abderhalden et al.* [9], and *Izumiya et al.* [10]. Its L-enantiomer was obtained by the interaction of brucine with the *N*-formyl derivative of the racemic compound [9] and, conveniently, by the stereospecific hydrolysis of its racemic amide with a hog-kidney amidase to yield L-*t*-butylglycine (**8**) and D-*t*-butylglycine amide [10]. Here, we report the preparation of L-*t*-butylglycine (**8**), its amide **3**, its N^{a}-*t*-butoxycarbonyl derivative **9**, and its methyl

[1]) Abbreviations are according to the IUPAC-IUB Commission on Biochemical Nomenclature (see [3]).

[2]) *J. L. Fauchère et al.*, to be published.

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ester **10** *via* an asymmetric *Strecker*-type synthesis according to *Patel & Worsley* [11]. Again, it was confirmed that the use of (*S*)-(−)-α-methylbenzylamine leads to the L-amino acid.

The main purpose of this work, however, was to provide a new enkephalin analogue containing Bug in the 'aliphatic' position 5 (which in nature is occupied by leucine as in [Leu^5]enkephalin, H · Tyr-Gly-Gly-Phe-Leu · OH, or by methionine as in [Met^5]enkephalin, H · Tyr-Gly-Gly-Phe-Met · OH [12], to assay its *in vitro* pharmacologic activity, and to try to correlate this activity, the steric, lipophilic, and enzyme-resistance characteristics of Bug.

We report the synthesis of [D-Ala^2, Bug^5]enkephalin amide (**7**) by a conventional approach with isolation and characterization of intermediate and final products. The *C*-terminal amide group was chosen because of synthetic convenience (its influence on the pharmacologic activity and opioid receptor-binding properties of the enkephalins has been well characterized [13]), and D-alanine was introduced, because [D-Ala^2]enkephalins [14] are expected to be more resistant towards aminopeptidase than the parent compounds with Gly.

We also report the partition coefficient of Bug in the system octanol/water as well as its *Hansch* π-value [15] as a measure of lipohilicity. Finally we describe the behaviour of **7** towards a number of proteolytic enzymes and give a preliminary report on its *in vitro* pharmacologic activity.

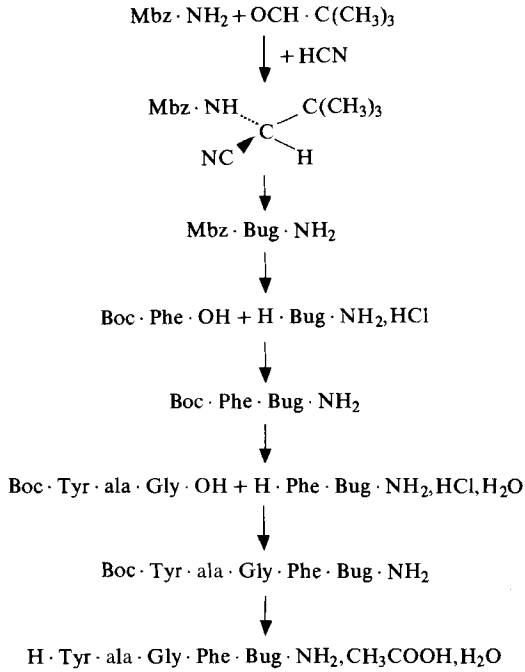
Asymmetric synthesis of t-*butylglycine.* The chiral center of the *Schiff* base formed from pivalaldehyde and (*S*)-(−)-α-methylbenzylamine served to induce the correct asymmetric addition of hydrogen cyanide to the double bond, resulting in a nearly quantitative optical yield of (*S*)-2-[(*S*)-α-methylbenzylamino]-3-dimethylbutyronitrile (**1**). The acidic hydrolysis of **1** in conc. sulfuric acid produced the amide **2** which, because of its two asymmetric centers, allowed further purification of the desired, major diastereoisomer. Catalytic hydrogenation led directly to the L-amino-acid amide **3**; the specific optical rotation of which was the same (but with opposite sign) as previously reported for the enzymatically produced D-enantiomer [10].

The optical purity and stereochemical identity of **3** was further checked by its hydrolysis with leucine aminopeptidase at pH 8.0 and in the presence of Mn^{++}-ions. The reaction was quantitative, thus almost certainly excluding a contamination of the substrate with the D-isomer. The free amino acid **8** showed the optical rotation reported [10]. It was converted to its N^{α}-Boc derivative **9** and its methyl ester **10** by classical procedures [16] [17], two new intermediates for peptide synthesis.

It was confirmed that **3** is extremely difficult to hydrolyse to **8** by acid hydrolysis [10].

*Synthesis of H · Tyr-ala-Gly-Phe-Bug · NH$_2$ (***7***, ala = D-Ala,* [D-*Ala*^{2}, *Bug*^{5}]-*enkephalin amide*) (see *Scheme*). Condensation of H · Bug · NH$_2$ (**3**) with Boc · Phe · OH according to *König & Geiger* [18] afforded Boc · Phe-Bug · NH$_2$ (**4**) which was subsequently deprotected in HCl/HCOOH to afford the dipeptide amide H · Phe-Bug · NH$_2$ (**5**). This was condensed by the same method with the fragment Boc · Tyr-ala-Gly · OH [19] to produce Boc · Tyr-ala-Gly-Phe-Bug · NH$_2$ (**6**) which was deprotected with trifluoroacetic acid to the final product {H · Tyr-ala-Gly-Phe-Bug · NH$_2$, CH$_3$COOH, H$_2$O} (**7**).

Scheme. *Synthesis of* [D-*Ala*^{2}, *Bug*^{5}]*enkephalin amide.* Bug = L-*t*-butylglycine; ala = D-Ala; Mbz = (−)-*S*-α-methylbenzyl



All the products mentioned were analytically characterized and pure by the criteria employed (*Tables 1–3*, see experimental part for HPLC., IR. and NMR. data).

Lipohilicity of Bug. In order to separate lipophilic from steric ('fatty' from 'bulky') effects of Bug on the properties of its enkephalin derivatives, the *Hansch* π-value [15] of its side-chain was determined using the equation π (side-chain) = log P (Bug) − log P (Gly). The partition coefficients P were measured in the system octanol/water by means of a ninhydrin assay [20] described for Bug in the experimental section. The substituent constant was calculated as π (Bug) = + 1.44 using log P (Gly) = − 3.21[3]) and the measured log P (Bug) = − 1.77 ± 0.11. Bug resembles methionine (π (Met) = + 1.34) more than leucine (π (Leu) = + 1.15) [21] as far as its lipophilic properties are concerned. This, despite the fact that the elemental composition of the Bug side-chain is the same as that of the Leu side-chain (C_4H_9), but quite different from that of the Met side-chain (C_3H_7S). The lipophilicity of Bug is reflected in the fact that during amino-acid analysis Bug emerges from the columns with the same retention time as Met.

Enzyme resistance of Bug peptides. – The intriguing question of whether the unnatural side-chain of Bug would make its pentapeptide derivative **7** more

[3]) *C. Hansch*, personal communication.

resistant to enzymic degradation *in vitro* was also investigated. To this end 0.4 mg of 7 and of several reference peptides *(Table 4)* were incubated at 37° with one of the peptidases and the degradation of the peptide followed by TLC. as a function of time. The semi-quantitative results can be interpreted as follows: Under conditions where [Met^5]enkephalin and [Leu^5]enkephalin are completely degraded (*i.e.* no more intact substrate can be detected) the Bug derivative 7 is left intact by thermolysine (pH 7.8) and by pepsin (pH 2.5). While D-Ala in position 2 protects the peptides against aminopeptidases, the introduction of Bug in position 5 increases the stability against carboxy peptidases such as thermolysine: no H · Bug · NH_2 can be detected under conditions where H · Leu · NH_2 and H · Met · NH_2 are easily split from the corresponding reference peptide. Among the five peptidases tested, α-chymotrypsin is the only one which degrades 7 about as easily as it degrades natural enkephalins. Another interesting observation is the fact that chloroacetyl-D,L-Bug · OH cannot be asymmetrically hydrolyzed by hog-kidney acylase I while H · Bug · NH_2 is readily cleaved in the presence of leucine aminopeptidase according to [10] and to our own experience.

Pharmacologic activity in vitro. Compound 7 was assayed in the classical guinea pig myenteric plexus/longitudinal muscle and mouse *vas deferens* assays [12] for its potency to depress electrically evoked contractions.

Prof. *Schiller* found an equal to slightly reduced potency of H · Tyr-ala-Gly-Phe-Bug · NH_2 (7) in the guinea pig ileum (GPI) assay according to [22] as compared to H · Tyr-ala-Gly-Phe-Met · NH_2, H · Tyr-ala-Gly-Phe-Leu · NH_2, and [Met^5]enkephalin.

Prof. *Kosterlitz* reported a 2–3 times increased potency of 7 in his GPI assay and – as expected for an amide – a somewhat reduced potency in the mouse *vas deferens* as compared to [Met^5]enkephalin and [Leu^5]enkephalin, respectively.

Beddell et al. [23] had discovered that in the GPI assay, among the β-branched amino acids in position 5, Val^5 reduced the potency very strongly, but Ile^5 did so only slightly. In view of these results, an analysis of the differential rôles of branching at the β-carbon, of bulk, lipophilicity, and protection against enzymic degradation provided by the amino acid in position 5 will require many more enkephalin analogues and assays. However, in view of the results obtained with adamantylalanine [1] [7] we feel that lipophilicity is one of the decisive factors governing potency from this position. We hope to report details in the near future.

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Experimental Part

General. Removal of solvents from dissolved compounds was carried out in a rotatory evaporator at reduced pressure (0.1–10 Torr) and low temperature ($t < 45°$). Condensation and deprotection as well as isolation of intermediates and products were mainly achieved by classical methods of peptide

chemistry (for details, see *Houben-Weyl* [24]). Amino-acid analyses were performed with a *Biotronik* BT6110 analyser and a *Hewlett Packard* 3388A automatic integrator. Specific rotations were measured with a *Perkin Elmer* 141 polarimeter. NMR. (δppm from TMS) and IR. (cm^{-1}) spectra were recorded with a *Varian* T60 and a *Beckman* IR33 spectrometer, respectively.

Reactants were purchased from *Fluka* AG, Buchs, CH, and proteolytic enzymes from *Serva* AG, Heidelberg, D. Product characteristics are displayed in *Tables 1–3.* Solvent ratios are in volume parts.

Partition coefficient (P). Four samples of the amino acid weighing between 0.3 and 2.5 mg were completely dissolved in 2 ml of water saturated with octanol, 25 ml of octanol saturated with water were then added to each sample and the partitioning carried out by shaking for 2 min. Centrifugation (2000 rpm, 20 min) afforded a complete separation of the phases. Aliquots of the aqueous phase were then treated with a standard ninhydrin solution (*Sigma* Chemicals) for 15 min in a boiling water-bath. After dilution with 5 ml of ethanol/water 1:1 the absorbance at 570 nm was recorded and the concentration of the amino acid calculated with the help of a calibration curve measured under the same

Table 1. *TLC.* t-*butylglycine and related compounds* (Rf values)[a])

Solvent		1	2	3	4	5	6	7	8	9	10
HE	[b])	0.50									
CM9	[c])		0.50		0.46	0.18					
CM1	[c])				0.68					0.56	0.52
CMA	[d])	0.85			0.50						
2BAW	[e])	0.65	0.44	0.28	0.72	0.43	0.64	0.52	0.21	0.78	0.38
BN	[f])	0.74		0.48	0.69		0.70	0.49	0.23		0.64
IWP	[g])			0.50			0.68	0.66			

[a]) Detected by I_2, ninhydrin, *Reindel-Hoppe* reagent [25], fluorescence quenching in UV., or other suitable means. Only one spot was observed on the *Merck* F254 silica gel plates. [b]) Hexane/ethyl acetate 4:1. [c]) $CHCl_3$/MeOH 9:1 or 1:1. [d]) $CHCl_3$/MeOH/CH_3CO_2H 95:5:3. [e]) 2-Butanol/CH_3CO_2H/water 72:7:21. [f]) Butanol/aqueous ammonia (25%) 10:3. [g]) 2-Propanol/water/pyridine 36:32:32.

Table 2. *Physical data of* t-*butylglycine and related compounds.*
Aspect: s = solid, c = crystalline (A). Solvent for crystallization (S). Yield (%). M.p. °C[a]). Specific rotation, $[\alpha]_D^{23}$ ($c = 1$, MeOH) unless noted.

	A	S	M.p.	Yield	$[\alpha]_D^{23}$ deg.
1	c	MeOH/H_2O	40	75	−203.9
2	c	EtOAc	113	81	− 88.9
3	c	EtOH/HCl[b])	205	80	+ 41.8[c])
4	s	Chrom.[d])	–	60	− 13.9
5	s	EtOH/Et_2O[e])	195–200 dec.	87	+ 16.3
6	s	Chrom.[f])	–	68	+ 11.7
7	s	Chrom.[g])	–	63	+ 19.8
8	c	H_2O/acetone	248 dec.	35	− 9.2[h])
					+ 9.0[i])
9	c	MeOH/Et_2O	165	70	− 7.2
10	c	2-propanol/diisopropyl ether	170	52	+ 17.0

[a]) Uncorrected. [b]) EtOH containing 1.1 equiv. HCl with respect to **3** base. [c]) +34.6 ($c = 3$, 5N HCl); [10]: +35. [d]) Chromatography on silica gel 40×2.2 cm in $CHCl_3$/MeOH 95:5. [e]) Precipitation. [f]) Chromatography on silica gel 40×3 cm in $CHCl_3$/MeOH 7:3. [g]) Chromatography first on *Sephadex* C25 75×2 cm in 1N CH_3CO_2H with a linear gradient of 0.1–0.4M ammonium acetate, then on *Sephadex* G10 20×1.5 cm in 0.1N CH_3CO_2H. [h]) $c = 3$, H_2O; [10]: −9.7°. [i]) $c = 3$, 5N HCl; [10]: +9.0.

Table 3. *Analytical data of* t-*butylglycine and related compounds.* Elementary composition (E); calculated molecular weight (M); microanalyses: [a]) C, H, N (% calc./% found).

	1	**2**	**3**[b])	**4**	**5**[c])
E	$C_{14}H_{20}N_2$	$C_{14}H_{22}N_2O$	$C_6H_{15}ClN_2O$	$C_{20}H_{31}N_3O_4$	$C_{15}H_{26}ClN_3O_3$
M	216.3	234.2	166.6	377.5	331.8
C	77.73/77.64	71.75/71.59	43.24/42.78	63.63/63.58	54.29/54.50
H	9.32/ 9.37	9.46/ 9.45	9.07/ 9.04	8.28/ 8.33	7.90/ 7.67
N	12.95/12.88	11.95/11.81	16.81/16.25	11.13/11.06	12.66/12.60
	6[d])	**7**[e])	**8**	**9**	**10**[f])
E	$C_{34}H_{48}N_6O_8$	$C_{31}H_{46}N_6O_9$	$C_6H_{13}NO_2$	$C_{23}H_{44}N_2O_4$	$C_7H_{16}ClNO_2$
M	668.8	646.7	131.2	412.6	181.7
C	61.06/60.77	56.18/55.89	54.94/54.67	66.95/66.67	46.28/46.55
H	7.24/ 7.25	7.00/ 6.98	9.99/ 9.92	10.75/10.62	8.88/ 8.73
N	12.57/11.95	12.68/12.67	10.68/10.42	6.79/ 6.73	7.71/ 7.86

[a]) Performed in the Laboratory of Organic Chemistry, ETHZ *(D. Manser).* [b]) Chloride analysis: 21.28/21.27. The low values for C, H, N might be due to a slight excess of HCl, but are unexplained. [c]) Chloride analysis: 10.68/10.36. The analyses are indicative of 1 equiv. of H_2O in the amorphous solid. [d]) Amino-acid analysis (Laboratory of Prof. *H. Zuber,* ETHZ) after hydrolysis with 6N HCl, 24 h, 110°: Tyr 1/0.99; Ala 1/1.06; Gly 1/1.05; Phe 1/0.95; Bug 1/1.00. [e]) Amino-acid analysis: Tyr 1/0.98; Ala 1/1.03; Gly 1/1.05; Phe 1/0.97; Bug 1/1.02. [f]) Chloride analysis: 19.52/19.12.

conditions without partitioning. The concentration in the organic phase was estimated as the difference to the total amount of solute. No concentration dependence was observed with Bug so that the mean value of P and its standard deviation were calculated from the 12 values corresponding to the 4 samples each at 3 different dilutions.

2-[(S)-α-Methylbenzylamino]-3,3-dimethylbutyronitrile (**1**). 1-[(*S*)-α-Methylbenzylimino]-2,2-dimethylpropane was prepared as an oil from 6.89 g (80 mmol) of pivalaldehyde and 10.6 g (88 mmol) of (*S*)-α-methylbenzylamine in 130 ml of ether at −10° in the manner described for 1-[(*S*)-α-methylbenzylimino]-2-(1-adamantyl)ethane [3]. The oil was immediately dissolved in 200 ml of absolute EtOH and the solution treated with 5.4 g (200 mmol) of liquid HCN using the manipulations described in [3]. Yield: 13 g of **1** *(Tables 1–3).* – IR.: 2200*m* (C≡N), 2960*s* (*t*-Bu), 3340*m* (N–H).

2-[(S)-α-Methylbenzylamino]-3,3-dimethylbutyramide (**2**). Following the sulfuric acid procedure [3], 4.32 g (20 mmol) of **1** were converted to **2**. The resulting mixture was carefully neutralized with 4N NaOH in the cold. The product was then extracted into ethyl acetate, washed as usual, isolated by evaporation of the solvent, and recrystallized: 3.8 g of **2** *(Tables 1–3).* – IR.: 1580*s* (C=O), 1675*s* (C=O), 2950*s* (*t*-Bu), 3340*m* (N–H). – ^{1}H-NMR. (D_6) DMSO: 0.85 (*s*, $C(CH_3)_3$); 1.20 (*d*, CH_3); 2.05 and 2.36 (2*s*, CH and NH, not identified); 3.48 (*qa*, CH); 7.05 (*s*, NH_2); 7.17–7.32 (*m*, C_6H_5).

L-(+)-t-*Butylglycine amide hydrochloride* (**3**). A solution of 1.17 g (5 mmol) **2** in 50 ml MeOH/CH_3CO_2H 4:1 was hydrogenated for 8 h at RT. and 760 Torr over 200 mg of 10% Pd/C. After replacing the used by the same amount of fresh catalyst, the hydrogenation was continued for 16 h. The mixture was filtered and the solvent was evaporated. Recrystallization of the residue from hot ethyl acetate in the presence of 1.1 equiv. (5.5 mmol) HCl yielded 670 mg of **3** *(Tables 1–3).*

t-*Butoxycarbonyl*-L-*phenylalanyl*-L-t-*butylglycine amide* (**4**). The condensation of 166.6 mg (1 mmol) of **3** with 265.2 mg (1 mmol) of *t*-butoxycarbonyl-L-phenylalanine was performed with 126 µl of *N*-ethylmorpholine, 136 mg of 1-hydroxybenzotriazole and 240 mg of dicyclohexylcarbodiimide in 25 ml of dimethylformamide following the usual procedure [18]. The impure crude product was purified by chromatography *(Table 2).* The amorphous residue (230 mg) was pure **4** *(Tables 1–3).*

L-*Phenylalanyl*-L-t-*butylglycine amide hydrochloride hydrate* (**5**). A solution of 377.4 mg of **4** in 30 ml of 0.1N HCl in HCO_2H was kept at RT. for 20 min, then diluted with 300 ml of ethyl acetate, and the solvents evaporated. The residue was dissolved in EtOH and precipitated with dry ether: 290 mg of **5** *(Tables 1–3).*

Table 4. *Enzymatic degradation* in vitro *of enkephalin analogues*[a])

	Thermolysine	Aminopeptidase M	Pronase	Pepsin	α-Chymotrypsin
Met-Enkephalin	+++	++++	++++	++++	+++
Leu-Enkephalin	++++	+	++++	++++	+++
Tyr-ala-Gly-Phe-Met-NH_2	+++	+	++	+	++
Tyr-ala-Gly-Car-Leu-NH_2	++++	+	++	+	+
Tyr-ala-Gly-Phe-Bug-NH_2	○	○	+	+	++++

[a]) Car = L-carboranylalanine [2]; ○ = no degradation is detectable on TLC.; +, ++, +++ = increasing partial degradation; ++++ = no more intact substrate detectable on TLC.

t-*Butoxycarbonyl*-L-*tyrosyl*-D-*alanyl-glycyl*-L-*phenylalanyl*-L-t-*butylglycine amide* (**6**). A solution of 331.8 mg (1.0 mmol) of **5** in 10 ml of dimethylformamide was neutralized with 130 µl of *N*-ethylmorpholine. The following compounds were then added in order: 409.4 mg (1 mmol) of *t*-butoxycarbonyl-L-tyrosyl-D-alanyl-L-glycine, 162 mg of 1-hydroxybenzotriazole and, after cooling to 0°, 248 mg of dicyclohexylcarbodiimide. After 1 h at 0° and 16 h at RT., the product was isolated in the usual manner and chromatographed for final purification *(Table 2)*. The purity of the fractions was determined with TLC. Yield: 452 mg of **6** *(Tables 1–3)*.

L-*Tyrosyl*-D-*alanyl-glycyl*-L-*phenylalanyl*-t-*butylglycine amide acetate hydrate* (**7**). A solution of 334 mg (0.5 mmol) of **6** in 5 ml of CF_3CO_2H was kept at RT. for 30 min and then evaporated under repeated addition of EtOH. The product was dissolved in MeOH, precipitated with ether, and then purified by chromatography. Yield: 210 mg of pure **7** *(Tables 1–3)*. The compound appeared to be homogeneous in high-pressure liquid chromatography (HPLC.) over *Lichrosorb* Si 60 (mean particle size 5 µm, column 3×250 mm) in the solvent mixture $CHCl_3$/MeOH starting with ratio 9:1 and increasing the relative volume of MeOH.

L-C^{α}-t-*Butylglycine* (t-*butylglycine, Bug,* t-*leucine, β-methylvaline*) (**8**). A solution of 500 mg (3 mmol) of **3** and 15 mg of $MnCl_2$, 4 H_2O in 50 ml of water was adjusted to pH 8 with 1N NaOH and treated with a cytosol containing 3.3 mg of leucine aminopeptidase (*Sigma* Chemicals). After 40 h at 37°, a few drops of CF_3CO_2H were added, whereupon the enzyme was denatured and eliminated by heating to 80° and filtration. The hydrolysis of the amide was complete (TLC.). The inorganic salts were removed by chromatography through a 60×1.5 cm column of *Sephadex* G10 in water and then by recrystallization: 138 mg of pure **8** *(Tables 1–3)*. – ^{1}H-NMR. (D_2O): 1.10 (*s*, $C(CH_3)_3$); 3.47 (*s*, CH) (peak ratio calc. 9.0:1, found 9.5:1), 4.72 (*s*, HDO), no other signal (purity criterion). Partition coefficient: log P(Bug) = −1.77±0.11.

t-*Butoxycarbonyl*-L-*butylglycine dicyclohexylamine salt* (**9**). A solution of 400 mg of **8** in 30 ml of 0.5N NaOH/dioxane 2:1 at 0° (apparent pH 12.5) was treated with 1 g of *t*-butyl dicarbonate. After 16 h at RT. most of the dioxane was removed by evaporation and the product extracted into ethyl acetate at pH 2. After evaporation of the solvent, the oily residue was dissolved in ether and treated with 0.6 ml of dicyclohexylamine. The precipitated salt was recrystallized to yield 870 mg of pure **9** *(Tables 1–3)*.

Methyl t-*butylglycinate* (**10**). A solution of 300 mg (2.3 mmol) of **9** in 4 ml of methanol was treated with 3 ml of thionyl chloride at 0°. After 40 h at 40° another addition of 3 ml of thionyl chloride was made, and the solution kept at 40° for another 24 h. The usual isolation procedure (with extraction into ethyl acetate at pH 9.5) yielded 220 mg of pure, recrystallized **10** *(Tables 1–3)*.

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