200. Synthesis and Biological Properties of Enkephalin-like Peptides Containing Carboranylalanine in Place of Phenylalanine[1])

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Summary

The syntheses of [D-Ala^2, Car^4, Leu^5]-enkephalin, its amide, and of [D-Ala^2, Car^4, Met^5]-enkephalin amide are described in detail. The three compounds show pronounced morphine-like potencies in certain bio-assays, and were used to investigate the influence of electronic, steric and hydrophobic properties of the side-chains of amino acids no. 4 and 5 on opioid activity (see [13]).

1. Introduction. – 'Fat' amino acids (see [3]) such as *o*-carboranylalanine (Car) [4] [5], adamantylalanine (Ada) [6], β-methylvaline (*t*-butylglycine, Bug) [7], and γ-methylleucine (neopentylglycine, Neo) [8] are artificial amino acids designed to combine steric bulk of their side-chains (a bulk corresponding roughly to that of rotational bodies derived from side-chain elements of the natural amino acids Phe, Val, and Leu), with enhanced lipophilicity [9] and aliphatic or aromatic characteristics. The concept of using such amino acids as probes for elucidating structure-activity relationships arose from the observation that the replacement of phenylalanine by Car in a chymotrypsin inhibitor [10] and in [Leu^5]-enkephalin [11] enhanced the respective enzyme and receptor affinity 3-fold.

Car, Ada, Bug, and – in order to enlarge the scope of the electronic characteristics – *p*-nitro-phenylalanine (Nip) [12], were recently employed in a study of electronic, steric, and hydrophobic factors influencing the action of enkephalin-like peptides, Tyr-ala-Gly-X-Y, on (μ-) opiate receptors [13]. It was found that the negative electromeric (-*E*) aromatic character of amino acid no. 4 (Phe in the natural peptide) and the hydrophobicity of amino acid no. 5 (Leu or Met in the natural product) were the dominant factors influencing both the pharmacological potency in the guinea pig ileum and the naloxone-like, stereospecific affinity for opiate receptors in rat brain homogenates. Quantitative analysis of the electronic, steric, and hydrophobic contributions to biological activity proves the heuristic and predictive value of such amino acid replacements [14].

[1]) Abbreviations are according to the *IUPAC-IUB Commission on Biochemical Nomenclature* [1] and *Houben-Weyl* [2]; or are specified in the text; Ala = L-alanine, ala = D-alanine.

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The synthesis and some biological properties of enkephalin-like peptides containing Car, Ada, and Nip in the positions 4 and 5 of [ala^2]-enkephalin [15] are described in this and the following two contributions [16] [17].

2. Syntheses. – The enkephalin-like pentapeptides HCl·H-Tyr-ala-Gly-Car-Y, with Y = Met-NH_2 (**7**), Leu-NH_2 (**10**), and Leu-OH (**13**) were prepared by classical solution methods. The *t*-butoxycarbonyl (Boc-) group [18] was used to protect amino functions. It was removed by acidolysis (HCl in formic acid or in ethyl acetate [19]) to produce the peptide hydrochlorides, directly. Where necessary, the carboxyl functions were protected as their methyl esters. Deprotection was effected by hydrolysis in mixtures of dioxane, methanol, and 2N NaOH for 12–15 min at room temperature. *N,N'*-Dicyclohexyl-carbodiimide [20] was used for peptide bond formation according to *König & Geiger* [21].

Intermediate and final products were analyzed for purity by TLC. on silica gel and by elemental analysis. Further proofs of identity were, in some cases, provided by amino-acid analysis and IR. spectroscopy. In the latter case, the band centered around 2580 cm^{-1} was indicative of the presence of B,H-bonds, and hence, of *o*-carboranylalanine [4].

The synthetic schemes were devised to minimize or even exclude epimerization [2]. Thus, the found $[\alpha]_D$-values are supposed to be characteristic of the expected diastereomers. In particular, alcaline hydrolysis did not seem to lead to extensive epimerization, because our tripeptide **3** showed – within limits – the same optical rotation as another preparation [22] synthesized without alcaline hydrolysis (see exper. part). Furthermore, the many pentapeptides prepared with our tripeptide are highly resistant towards the action of aminopeptidase [13], thus excluding the presence of L-alanine.

Scheme 1 outlines our modified synthesis of the known *N*-terminal tripeptide fragment Boc-Tyr-ala-Gly-OH (**3**) [22].

Scheme 1

H-ala-OH + Me_3C-O-CO-O-CO-O-CMe_3 [23] → Boc-ala-OH (**1**) [24]

$$\xrightarrow{\text{HCl·H-Gly-OMe}} \text{Boc-ala-Gly-OMe} \rightarrow \text{HCl·H-ala-Gly-OMe} \xrightarrow{\text{Boc-Tyr-OH}}$$

Boc-Tyr-ala-Gly-OMe (**2**) → Boc-Tyr-ala-Gly-OH (**3**) [22]

Scheme 2 illustrates the synthesis of HCl·H-Car-Met-NH_2 (**5**) and its condensation with **3** to produce the pentapeptide amides **6** and **7**, sequentially. The educt Boc-Car-OH was prepared according to [5]. By using HCl·H-Leu-NH_2 or HCl·H-Leu-OMe as reactants instead of HCl·H-Met-NH_2, the same procedure was employed for the preparation of Boc-Car-Leu-NH_2 (**8**), Boc-Tyr-ala-Gly-Car-Leu-NH_2 (**9**), and HCl·H-Tyr-ala-Gly-Car-Leu-NH_2 (**10**), as well as Boc-Car-Leu-OMe (**11**) [5], Boc-Tyr-ala-Gly-Car-Leu-OMe (**12**), and HCl·H-Tyr-ala-Gly-Car-Leu-OH (**13**). However, in the latter case, the step **12** → **13** included alcaline ester hydrolysis before the acidolytic removal of Boc.

Scheme 2

Boc-Car-OH [5] + HCl · H-Met-NH_2 → Boc-Car-Met-NH_2 (**4**) → HCl ·

H-Car-Met-NH_2 (**5**) $\xrightarrow{3}$ Boc-Tyr-ala-Gly-Car-Met-NH_2 (**6**) → HCl · H-Tyr-ala-Gly-Car-Met-NH_2 (**7**)

3. Biologic activity. – Compounds **7**, **10** and **13** were investigated for their enkephalin- and morphine-like action with two *in vitro* systems[2]): (i) the inhibition of electrically evoked contractions of the guinea pig ileum longitudinal muscle-myenteric plexus preparation ('guinea pig ileum', GPI, see [13]), and (ii) [^{3}H]naloxone displacement from its binding sites in rat brain cell membrane preparations ('stereospecific affinity', NAL, see [13]). The potencies were estimated from log dose-response plots as the reciprocals of the peptide concentrations necessary for causing 50% of the maximal effect ($1/IC_{50}$) in each case.

Furthermore, **7** and **10** were assayed for their *in vivo* analgesic action in the mouse tail flick test after intracerebro-ventricular application (courtesy of Dr. *J. Pless, Sandoz AG.*, Basel).

The following *in vitro* potencies relative to H-Tyr-ala-Gly-Phe-Met-NH_2 (= 1.0) were found: 1.0 ± 0.4 (**13**), 1.9 ± 0.2 (**10**), and 3.2 ± 1.5 (**7**) in the GPI test, and 0.7 (**13**), 1.5 (**10**), and 5.8 (**7**) in the NAL test. Considering the fact that H-Tyr-ala-Gly-Phe-Leu-OH and its amide are only about ⅓ as potent as H-Tyr-ala-Gly-Phe-Met-NH_2, the enhancements caused by the replacement of Phe with the pseudoaromatic Car are quite considerable in both tests, at least 2-fold. As shown in a more detailed comparative analysis, this potentiation is most likely due to the pronounced electrophilic aromatic property *(-E)* of the carborane moiety, and not to its hydrophobic and space-filling ('fat') character [13].

In vivo, however, the analgesia produced by **7** and **10** in 10 μg doses was weaker and of shorter duration than that caused by H-Tyr-ala-Gly-Phe-Met-NH_2 in the same dose. Which factors – degradation, distribution, or others – are responsible for this reduction, is still unclear.

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Experimental Part

General. – Melting points (m.p.) were determined in open capillaries and are uncorrected. For the preparation of protected amino acids and standard procedures in peptide synthesis, see [2]. TLC. was carried out on *Merck* silica gel plates. The spots were revealed by standard techniques (UV., ninhydrin, *Pauli* and *Reindel-Hoppe* reagents, iodine, *etc.*). The solvents of the TLC. systems were: A = acetic acid, B1 = 1-butanol, B2 = 2-butanol, C = chloroform, E = ethyl acetate, H = hexane, I = 2-propanol, M = methanol, N = aqueous ammonia (25%), P = pyridine, W = water. Solvent ratios are in volume parts. Evaporations were carried out in rotatory evaporators at reduced pressure (0.1–10 Torr) and low temperature (35°). Unless otherwise indicated, acidifications were performed with an aqueous solution containing

[2]) Courtesy of Prof. Dr. *P. W. Schiller*, Laboratoire de Recherches sur les Polypeptides, Institut de Recherches Cliniques de Montréal, Montréal, Canada.

10% by weight of both $KHSO_4$ and K_2SO_4 (pH 2). Extraction into organic solvents was followed by appropriate steps, such as washing with hydrogencarbonate, acid, and water, drying, and evaporation, as usual. RT. is room temperature, about 20°. Abbreviations: DMF = *N,N*-dimethylformamide, DCCI = *N,N'*-dicyclohexylcarbodiimide, DCU = *N,N'*-dicyclohexylurea.

Preparation of Boc-ala-OH (**1**). A solution of D-alanine (10 g, 112 mmol) in dioxane/water 2:1 was neutralized with 1 N NaOH (112 ml), cooled to about 0°, treated at this temperature with bis(*t*-butyl)dicarbonate [23], and kept for 20 h at RT. After evaporation of excess dioxane, the solution was acidified, and the product extracted into ethyl acetate and isolated as usual. Recrystallization from ethyl acetate/hexane afforded 19.2 g (90%) of pure **1**. TLC., Rf 0.60 (C/M 1:1), 0.43 (C/M/A 95:5:3). M.p. 81°. $[\alpha]_D^{20} = +23.1$ ($c = 1$, MeOH).

$C_8H_{15}NO_4$ (189.2) Calc. C 50.79 H 7.99 N 7.40% Found C 50.83 H 8.14 N 7.27%

Preparation of Boc-Tyr-ala-Gly-OMe (**2**). A solution of **1** (1.89 g, 10 mmol) and H-Gly-OMe·HCl (1.26 g, 10 mmol) in DMF (15 ml) was treated according to the procedure of *König & Geiger* [2] [21] with 10 mmol each of *N*-ethylmorpholine and 1-hydroxy-benzotriazole at RT., followed by DCCI (11 mmol) at 0°. Boc-ala-Gly-OMe was obtained as an oil, homogeneous on TLC., Rf 0.64 (C/M/A 95:5:3). It was dissolved in ethyl acetate containing HCl (2N, 12 ml) and kept thus at RT. for 45 min. Evaporation gave solid, very hygroscopic H-ala-Gly-OMe·HCl; TLC. Rf 0.16 (B2/A/W 10:1:3), 0.21 (C/M 1:1). This product was dissolved, together with Boc-Tyr-OH (2.15 g, 7.6 mmol), *N*-ethylmorpholine (0.96 ml), and 1-hydroxybenzotriazole (2.1 g) in dimethylformamide (10 ml). Condensation was effected with *N,N'*-dicyclohexylcarbodiimide (1.7 g) at 0° (2 h) and RT. (15 h) as above. Evaporation, acidification, extraction into ethyl acetate, washing sequentially with hydrogencarbonate and acid, drying, evaporation, and crystallization from ethyl acetate/diethylether yielded 2.41 g (57%) of pure **2**. TLC. Rf 0.64 (B2/A/W 10:1:3), 0.64 (B2/N 10:3). M.p. 156° (dec.), $[\alpha]_D^{20} = +39.6°$ ($c = 1$, MeOH).

$C_{20}H_{29}N_3O_7$ (423.5) Calc. C 56.72 H 6.90 N 9.96% Found C 56.66 H 6.96 N 9.83%

Preparation of Boc-Tyr-ala-Gly-OH (**3**). Compound **2** (1.27 g, 3 mmol) was dissolved in dioxane/methanol/2 N NaOH 1:1:1 (18 ml) at RT. Hydrolysis of the ester group was complete after 12 min as judged by TLC. After neutralization with 0.1 N HCl and evaporation of the organic solvents, the remaining mixture was acidified and the product extracted into ethyl acetate. Isolation as usual. Precipitation from ethyl acetate with hexane afforded 0.93 g (76%) of **3** as a pure, colourless powder. TLC., Rf 0.53 (B2/A/W 10:1:3), 0.19 (B2/N 10:3). M.p. 110–115° (dec.), $[\alpha]_D^{20} = +39.6°$ ($c = 1$, MeOH), ([22] reports +41.6°, same conditions).

$C_{19}H_{27}N_3O_7$ (409.4) Calc. C 55.73 H 6.75 N 10.26% Found C 55.44 H 6.94 N 9.89%

Preparation of Boc-Car-Met-NH$_2$ (**4**). Boc-Car-OH [5] (166 mg, 0.5 mmol), H-Met-NH$_2$·HCl (92 mg, 0.5 mmol), and *N*-ethylmorpholine (58 mg, 0.5 mmol) were dissolved in dry DMF at RT. Condensation was effected by the addition at 0° of 1-hydroxybenzotriazole (135 mg, 1 mmol) and DCCI (115 mg, 0.55 mmol). After 1 h at 0° and 16 h at RT., one drop of acetic was added to the refrigerated mixture, and the DCU removed by filtration. The filtrate was evaporated, the residue dissolved in a small amount of methanol and filtered through a mixed-bed ion exchanger (*Amberlyst* 15 plus *Amberlyst* A-21). The eluate was evaporated and the residue triturated with hexane until a solid, colourless powder had resulted. This represented pure **4** (185 mg, 80% yield). M.p. 80–82° (dec.). TLC.: Rf 0.59 (C/M/E 95:5:3), 0.69 (B2/A/W 10:1:3). $[\alpha]_D^{20} = -42.2°$ ($c = 0.6$, $CHCl_3$). Diagnostic IR. resonances in $CHCl_3$ (cm^{-1}): 3400 (N–H), 2960 (C–H), 2580 (B–H), 1690, 1670 (amide).

$C_{15}H_{35}B_{10}N_3O_4S$ (461.64) Calc. C 39.03 H 7.64 N 9.10% Found C 39.18 H 7.76 N 9.01%

Preparation of H-Car-Met-NH$_2$·HCl (**5**). Compound **4** (175 mg, 0.38 mmol) was dissolved in HCl/HCOOH (0.12 N, 4 ml) and mercaptoethanol (0.5 ml). After 15 min at RT., the solvents were evaporated and the product dissolved in ethyl acetate. Purification by chromatography over silica gel, using ethyl acetate/ethanol 15:1 as the eluant. The fractions containing pure material (TLC.) were combined and evaporated. Precipitation from ethyl acetate with pentane afforded pure **5** as an amorphous solid (110 mg, 73% yield). M.p. 103–104° (dec.). TLC.: Rf 0.23 (C/M/E 95:5:3), 0.59 (B2/A/W 10:1:3). $[\alpha]_D^{20} = +31.0°$ ($c = 0.5$, $CHCl_3$). Diagnostic IR. resonances in $CHCl_3$ (cm^{-1}): 3310–3430 (N–H), 2950 (C–H), 2580 (B–H), 1690, 1660 (amide).

$C_{10}H_{28}B_{10}ClN_3O_2S$	Calc.	C 30.18	H 7.09	N 10.56	S 8.06%
(397.98)	Found	„ 30.11	„ 7.19	„ 10.38	„ 8.23%

Preparation of Boc-Tyr-ala-Gly-Car-Met-NH_2 (**6**). Tripeptide **3** (103 mg, 0.25 mmol) and dipeptide **5** (100 mg, 0.25 mmol) were condensed according to the procedure of *König & Geiger* [2] [21] using the same molar ratios of the other additives as for the synthesis of **4** and DMF/pyridine 2:1 (3 ml) as solvent. The crude product was purified as described for **4**, and then crystallized from ethyl acetate/pentane. The yield of pure **6** was 132 mg (70%). TLC.: Rf 0.15 (C/M/E 95:5:3), 0.76 (B2/A/W 10:1:3). M.p. 147–150° (dec.). IR. (nujol) showed the diagnostic B–H resonance at 2580 cm^{-1}.

$C_{29}H_{52}B_{10}N_6O_8S$ (752.95) Calc. C 46.26 H 6.96 N 11.15% Found C 46.51 H 7.18 N 10.82%

Preparation of H-Tyr-ala-Gly-Car-Met-NH_2·HCl (**7**). The Boc-group was removed as described for the preparation of **5**. A solution of **6** (113 mg, 0.15 mmol) in 0.12 N HCl in HCOOH (2 ml) and mercaptoethanol (0.2 ml) yielded, upon evaporation and crystallization from methanol/diisopropylether/pentane (but without chromatography), pure **7**, that was recrystallized from the same solvents. Yield 90 mg (87%). M.p. 153–156° (dec.). TLC., Rf 0.53 (B2/A/W 10:1:3), 0.71 (B1/P/A/W 50:12:12:25). $[\alpha]_D^{25} = +2.0°$ ($c = 0.35$, MeOH). – UV. (ethanol) $\lambda(max) = 278$ nm ($\varepsilon = 1452$). – IR. (nujol) showed the diagnostic B–H resonance at 2580 cm^{-1}. – Amino acid analysis: Tyr 0.92, Ala 0.95, Gly 1.00 (reference), Met 0.90, Car is not resolved.

$C_{24}H_{45}B_{10}ClN_6O_6S$ (689.29) Calc. C 41.82 H 6.58 N 12.19% Found C 41.57 H 6.43 N 11.77%

Preparation of Boc-Car-Leu-NH_2 (**8**). Boc-Car-OH [5] (2 mmol) and H-Leu-NH_2·HCl (2 mmol) were condensed according to the procedure described for Boc-Car-Met-NH_2 (**4**), using 2 mmol each of *N*-ethylmorpholine and 1-hydroxybenzotriazol, 2.2 mmol of DCCI, and 20 ml of dry DMF. The product, after removal of the DCU and the solvents, was isolated by extraction into ethyl acetate, as usual. It was recrystallized from ethanol/pentane mixtures. Yield 160 mmol (80%) of pure **8**. TLC.: Rf 0.61 (C/M 9:1), 0.72 (B2/A/W 10:1:3), 0.53 (E/M 5:1). M.p. 118° (dec.). $[\alpha]_D^{23} = -36.3°$ ($c = 1$, MeOH).

$C_{16}H_{37}B_{10}N_3O_4$ (443.8) Calc. C 43.31 H 8.41 N 9.47% Found C 43.61 H 8.63 N 9.07%

Preparation of Boc-Tyr-ala-Gly-Car-Leu-NH_2 (**9**). In order to remove the Boc-group **8** (1.1 mmol) was dissolved in 0.4 N HCl in HCOOH (1.2 mol-equiv.). After 20 min at RT., the solvent was evaporated and the residue triturated five times with dry ethyl ether. The H-Car-Leu-NH_2·HCl, thus obtained, was condensed with Boc-Tyr-ala-Gly·OH **3** as described for the preparation of Boc-Car-Leu·NH_2 **8**, and isolated in the same manner. The crude product contained som DCU. This was removed by chromatography over silica gel (2 × 45 cm) with $CHCl_3/CH_3OH$ 95:15. Recrystallization from ethanol/pentane yielded pure **9** (0.7 mmol). TLC.: Rf 0.19 (C/M 9:1), 0.77 (B2/A/W 10:1:3), 0.30 (E/M 5:1). M.p. 165° (dec.) $[\alpha]_D^{23} = -0.9°$ ($c = 1$, MeOH).

$C_{30}H_{54}B_{10}N_6O_8$ (734.9) Calc. C 49.02 H 7.41 N 11.44% Found C 49.33 H 7.65 N 11.00%

Preparation of H-Tyr-ala-Gly-Car-Leu-NH_2·HCl (**10**). The Boc-group of **9** (0.5 mmol) was removed with HCl/HCOOH exactly as described for Boc-Car-Leu-NH_2 in the preparation of **9**. The pure product **10** (0.45 mmol) crystallized from water. TLC.: Rf 0.52 (B2/A/W 10:1:3), 0.70 (I/W/P 7:6:6). M.p. 165–170° (dec.). $[\alpha]_D^{23} = +2.6°$ ($c = 1$, MeOH).

$C_{25}H_{47}B_{10}ClN_6O_5 \cdot H_2O$ (689.3)	Calc.	C 43.56	H 7.16	N 12.19	Cl 5.14%
	Found	„ 43.87	„ 7.30	„ 11.99	„ 5.10%

Preparation of Boc-Tyr-ala-Gly-Car-Leu-OMe (**12**). Removal of the Boc-group from **11** [5] and condensation of the resulting dipeptide methyl ester hydrochloride with **3** was effected exactly as described for the amide **9**. The pentapeptide derivative **12** was isolated and purified in the same manner, except that it was recrystallized from ethyl acetate/diethyl ether. Yield 75% of pure **12**. TLC.: Rf 0.29 (C/M 9:1), 0.77 (B2/A/W 10:1:3), 0.70 (E/M 5:1). M.p. 169° (dec.). $[\alpha]_D^{23} = -12.7°$ ($c = 1$, MeOH).

$C_{31}H_{55}B_{10}N_5O_9$ (749.7) Calc. C 49.64 H 7.39 N 9.34% Found C 49.76 H 7.36 N 9.25%

Preparation of H-Tyr-ala-Gly-Car-Leu-OH·HCl (**13**). Compound **12** (1.5 mmol) was hydrolyzed during 15 min at RT. in dioxane/methanol/1 N NaOH 1:1:1 (10 ml). The product, Boc-Tyr-ala-Gly-Car-Leu-OH, was isolated as described for **3**. The crude material was then treated with HCl/HCOOH exactly as described for **10**, and isolated in the same manner, except that it was crystallized (as hydrochloride) from ethanol/pentane. Yield 1.2 mmol of pure **13**. TLC.: Rf 0.20 (C/M 9:1), 0.75 (B2/A/W 10:1:3), 0.81 (1/W/P 7:6:6). M.p. 158° (dec.). $[\alpha]_D^{23} = -4.9°$ ($c = 1$, MeOH).

$C_{25}H_{46}B_{10}ClN_5O_7$ (672.2)	Calc.	C 44.67	H 6.90	N 10.42	Cl 5.27%
	Found	„ 44.32	„ 7.02	„ 10.13	„ 5.07%

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