Synthesis of human des-tryptophan-1,norleucine-12-minigastrin-II and its biological activities

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A human minigastrin-II analog was prepared by conventional methods in solution using *N*-benzyloxycarbonyltyrosine *O*-sulfate as starting material in the synthetic route. Upon removal of the acid-labile protecting groups and purification by preparative reversed-phase chromatography the sulfated gastrin peptide was obtained in satisfactory overall yields as homogeneous material. It was found to be about twice as active as the non-sulfated form in stimulating gastric acid secretion and to exhibit a 10-fold higher affinity to gastrin receptors of purified parietal cells.

Gastrin *Sulfation* *Peptide synthesis* *Gastric acid secretion*

1. INTRODUCTION

The hormone gastrin is known to exhibit both in circulation and in tissues of origin a pronounced heterogeneity regarding the peptide length and the sulfation of the single tyrosine residue present in the C-terminal sequence [3]. Extensive comparative studies have been performed on the various gastrins in respect to their peptide length using mainly synthetic preparations and related analogs. Conversely, investigations on the sulfated forms were limited by the low amounts of material available from natural sources. Attempts to produce the sulfated gastrin via direct sulfation of the phenolic function in synthetic human little-gastrin-I with pyridine-SO_3 failed [4]. As expected, side reactions were found to occur to significant extents at various reactive groups of the gastrin sequence.

We have recently proposed a new approach for the synthesis of tyrosine *O*-sulfate-containing peptides [5,6]. It is based on the direct use of suitable derivatives of this amino acid as starting materials and it has been successfully applied for the synthesis of cholecystokinin-related peptides [7,8]. This new strategy has now been adopted for the elaboration of clean syntheses of sulfated gastrin peptides.

Here, the synthesis of a sulfated gastrin analog, i.e. human des-tryptophan-1,norleucine-12-minigastrin-II, is described. From previous structure-function studies it is known that the methionine residue of the C-terminal sequence can be replaced by leucine or norleucine and the N-terminal tryptophan residue deleted without any effect on the potency of human minigastrin-I [9,10].

Standard abbreviations as recommended by the IUPAC-IUB commission on Biochemical Nomenclature are used. For gastrins abbreviations according to [1,2] are used, e.g. HG13-II = C-terminal tridecapeptide of gastrin sulfated at the tyrosine residue

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2. MATERIALS AND METHODS

Amino acid derivatives used in the synthesis were prepared according to [11]. For preparative HPLC Jobin-Yvon Prep-10 was used. TLC was carried out on HPTLC silica gel 60 plates (Merck) and compounds were visualized by chlorine/toluidine and Pauly reagents.

Acid hydrolyses were performed in 6 M HCl containing 2.5% thioglycolic acid at 110°C for 24 h in evacuated sealed ampoules, and the aminopeptidase M digestion in Tris-HCl buffer (pH 7.8) at 37°C for 24 h. Amino acid analyses were obtained on a Biotronic amino acid analyser (model LC 6001). The gas chromatographic racemization test on a Chirasil-Val glass capillary column was carried out according to Frank et al. [12].

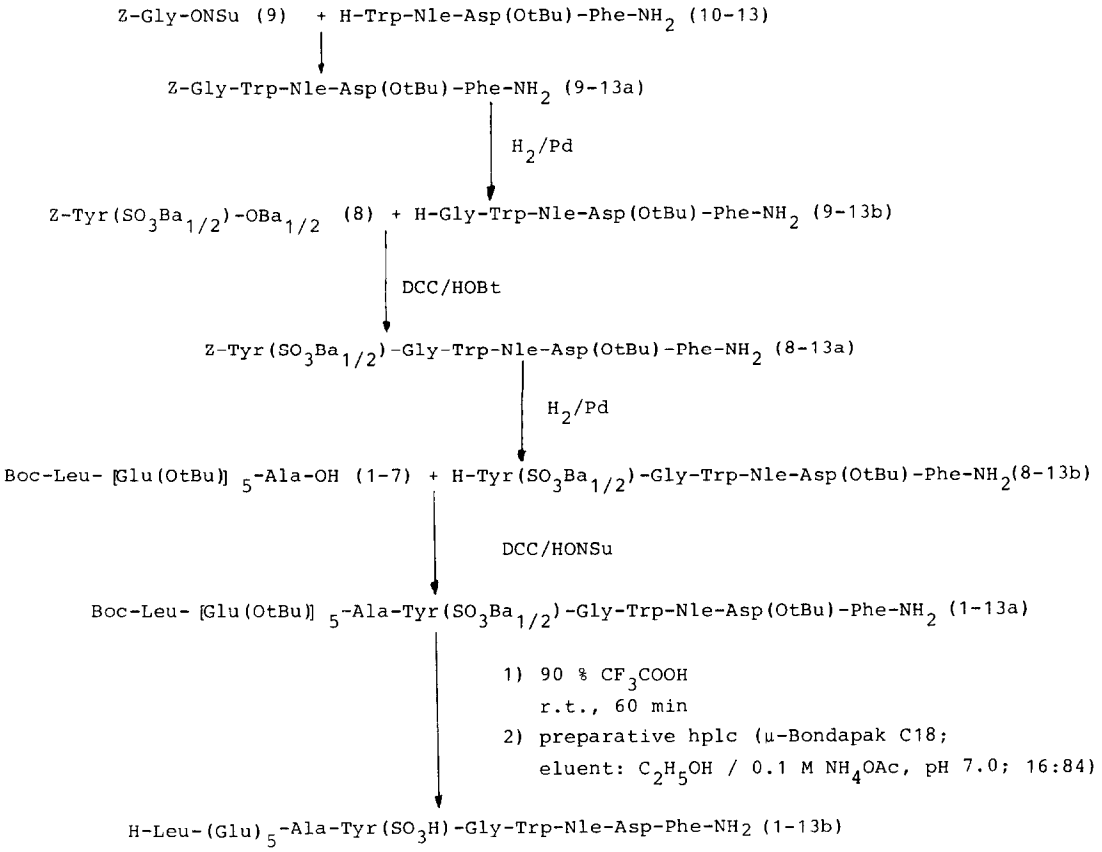
Stimulation of gastric acid secretion was determined in anaesthetized rats (~300 g) according to the procedure of Gosh and Schild [13] modified by Pham-Tham-Chi et al. [14].

3. RESULTS

3.1. *Synthesis of human des-tryptophan-1,norleucine-12-minigastrin-II (Nle^{11}-HG13-II)*

Taking advantage of our previous synthetic studies on human minigastrin-I analogs [10,15], the synthesis of Nle^{11}-HG13-II was performed according to the Schwyzer-Wünsch strategy using the fragment condensation procedure.

Following the synthetic route outlined in scheme 1 the side chain protected C-terminal tetrapeptide amide (**10–13**) [8] was acylated with Z-Gly-ONSu (**9**) to yield the N^{α}-benzyloxycarbonylpentapeptide derivative (**9–13a**) which in turn was deprotected



Scheme 1. Synthetic route for Nle^{11}-HG13-II.

at the α-amino function by hydrogenolysis over Pd catalyst for further chain elongation. The tyrosine *O*-sulfate residue (**8**) was then incorporated via condensation of the pentapeptide derivative (**9–13b**) with *N*-benzyloxycarbonyl derivative barium salt [6] by means of DCC in the presence of 1-hydroxybenzotriazole [16] as previously reported for the cholecystokinin peptides [6–8]. Both the high yield and the homogeneity of the resulting hexapeptide derivative hemibarium salt (**8–13a**) confirm the usefulness and high stability of the tyrosine *O*-sulfate moiety. Subsequent hydrogenolysis led in 80% yield over the two steps to the N^{α}-deprotected hexapeptide (**8–13b**) as key intermediate for the final condensation with the suitably protected N-terminal fragment (**1–7**) which was prepared in the usual manner mainly via *N*-hydroxysuccinimide esters: this synthetic step – via DCC in the presence of *N*-hydroxysuccinimide [17] – produced the fully protected tridecapeptide derivative hemibarium salt (**1–13a**) in 75% yield [amino acid analysis of the acid hydrolysate: Asp, 1.03 (1); Glu, 4.72 (5); Gly, 1.00 (1); Ala, 0.92 (1); Leu, 0.92 (1); Nle + Tyr, 1.99 (2); Phe, 1.04 (1);

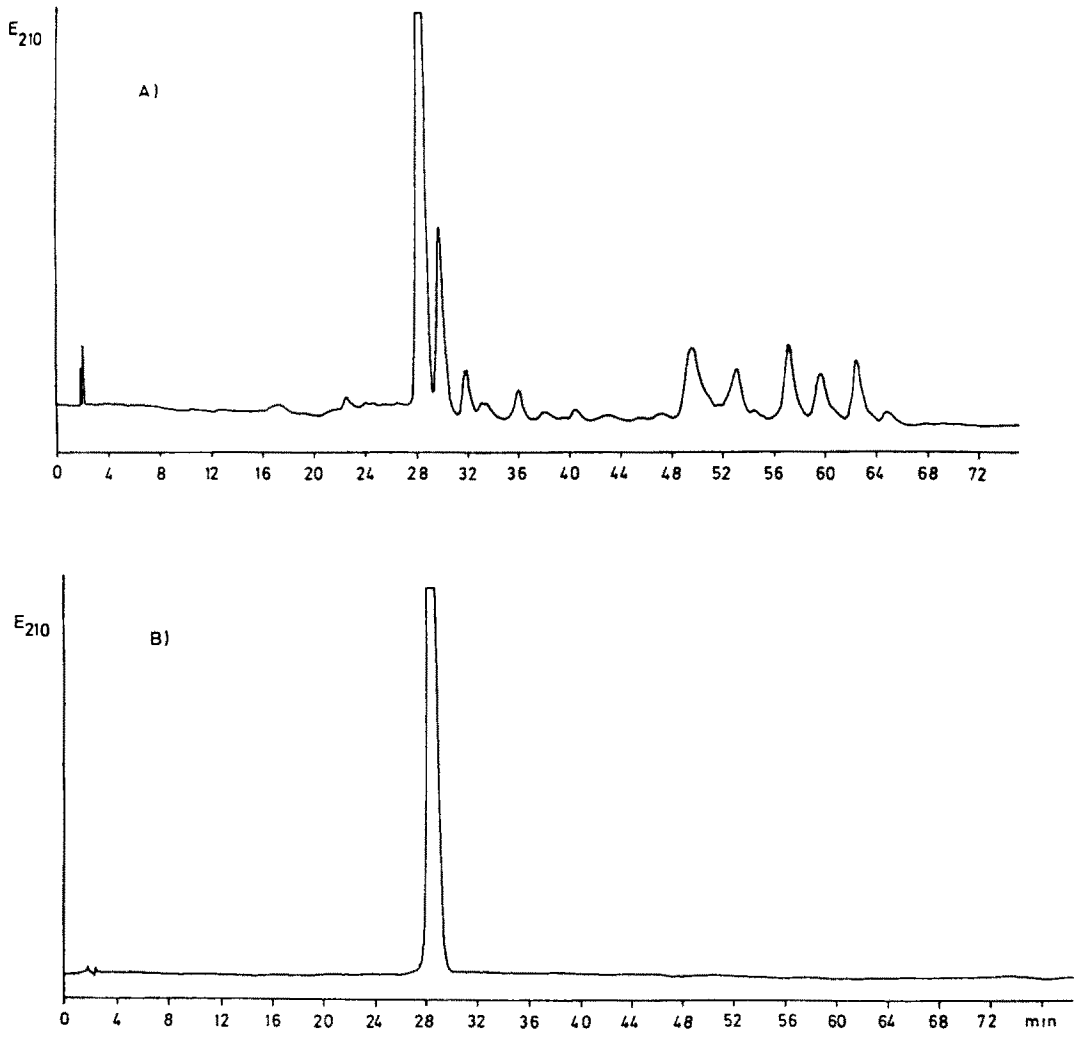


Fig.1. HPLC of Nle[11]-HG13-II. (A) Crude product, (B) purified product. Column: μ-Bondapak C18 (30 × 0.4 cm); eluent; acetonitrile/0.1 M sodium phosphate buffer (pH 5.4). Linear gradient from 15 to 28% acetonitrile in 80 min; flow rate: 1.7 ml/min.

Trp, 1.02 (1); peptide content: 91% calcd for M_r = 2213.2]. The integrity of the tyrosine *O*-sulfate moiety was assessed throughout the synthesis by IR spectroscopy (ν = 1050 cm^{-1}) as well as by TLC (Pauly test: negative).

For removal of the acid-labile protecting groups compound (**1–13a**) was exposed to 90% trifluoroacetic acid in the presence of 2-methylindole as scavenger. As expected from previous studies [5] partial hydrolysis of the sulfate monoester occurred in the range of 10–15% (see fig.1A: desulfated Nle^{11}-HG13 at 30 min retention time). *tert*-Butylation of the tryptophan residue [18] was again found to occur to significant extents (fig.1A: byproducts with higher retention time) even in aqueous trifluoroacetic acid. The latter conditions were reported to suppress this side reaction [19]. The crude deprotection product was purified on μ-Bondapak C18 (4 × 40 cm) using ethanol/0.1 M ammonium acetate buffer (pH 7.0) (16:84, v/v) at a flow rate of 18 ml/min as eluent. Upon Millipore filtration and repeated freeze-drying from 1% ammonia Nle^{11}-HG13-II (**1–13b**) was obtained in 39% yield (based on compound (**1–13a**) and on the peptide content of 86%); the product behaved homogeneously on HPTLC (solvent systems: *n*-butanol/acetic acid/pyridine/water, 60:6:20:24 and 60:6:40:24) and HPLC (fig.1B) and exhibited the expected amino acid ratios in the acid hydrolysate [Asp, 1.00 (1); Glu, 4.83 (5); Gly, 1.00 (1); Ala, 1.00 (1); Leu, 1.01 (1); Nle + Tyr, 1.95 (2); Phe, 1.05 (1); Trp, 0.89 (1); peptide content: 86% calcd for M_r = 1709.8] and AP-M digest [Asp, 1.01 (1); Glu, 4.84 (5); Gly, 1.01 (1); Ala, 1.00 (1); Leu, 1.03 (1); Phe, 1.01 (1); Trp, 1.01 (1); Tyr (SO_3H), 1.03 (1); Tyr, 0.00 (0); Nle, 1.01 (1); recovery: 84.5% for M_r = 1709.8]. The gas chromatographic racemization test excludes racemization along the synthesis to greater extents (D-Ala 1.5%; D-Leu < 1%; D-Asp 2.4%; D-Phe 1.1%; D-Glu 2.2%; D-Tyr 0.9%; D-Trp < 1%; D-Nle <1%); IR(KBr): ν = 1050 cm^{-1}.

3.2. *Biological activities of Nle^{11}-HG13-II*

Stimulation of gastric acid secretion by sulfated and non-sulfated Nle^{11}-HG13 was comparatively determined in male rats. The experimental results (table 1) indicate for the sulfated gastrin peptide a significant enhancement in potency (about twice as potent as the non-sulfated gastrin) and even more surprisingly, also in efficacy.

Table 1

Gastric acid secretion in rats (300 g) after bolus i.v. injection of gastrin peptides (saline solution) expressed in μmol H^+/40 min (mean ± SD)

Dose (pmol/kg)	Response to	
	Nle^{11}-HG13-I	Nle^{11}-HG13-II
50	5.5 ± 0.8 (n = 12)	11.5 ± 4.3 (n = 4)
150	10.9 ± 1.0 (n = 42)	17.0 ± 7.0 (n = 6)
300	15.2 ± 1.1 (n = 60)	30.0 ± 7.6 (n = 7)
450	18.1 ± 2.2 (n = 26)	n.d.

Additionally, Nle^{11}-HG13-I and Nle^{11}-HG13-II were tested for their ability to inhibit binding of ^{125}I-Leu^{25}-HG17-I to isolated rabbit purified parietal cells known to contain high-affinity gastrin receptors. The sulfated gastrin peptide exhibited a 10-fold higher binding affinity in this experimental model than the nonsulfated form (the detailed experimental work will be reported elsewhere).

4. DISCUSSION

The few comparative studies reported so far on sulfated and non-sulfated human little-gastrin seem to indicate that sulfation does not influence their effects on gastric parietal cells and central neurons nor the clearance rates [1,20–22]. Conversely, sulfation of short gastrin-related peptides was found to enhance their potency; this fact was mainly attributed to an increased resistance to metabolic clearance [23]. Our results concerning in vivo gastric acid secretion triggered by bolus i.v. injection of the sulfated and non-sulfated minigastrin analog contrast with the above findings and interpretation. The higher potency and particularly the efficacy of the hormone upon sulfation cannot be attributed only to a lowered hepatic uptake and clearance. Sulfation also affects the affinity of the hormone for the target cells in the stomach as was confirmed by competitive in-

hibition studies on binding of radiolabeled gastrin to parietal cells. These results clearly indicate that sulfation of gastrin must play a significant biological role. To unravel its physiological importance further detailed studies will be performed with the synthetic gastrin-II analog.

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