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The synthesis of a small library of prospective growth hormone secretagogues

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Employing tools of combinatorial chemistry, an original methodological approach has been developed and applied for the design and synthesis of a small library of peptide-like compounds, prospective growth hormone (GH) secretagogues. For this purpose seven building blocks of tBoc- and Fmoc-protected amino acids was used. In this way, a small, tripeptoid library on polyethylene glycol monomethyl ether 5000 (PEG 5000) as a soluble support was obtained. The library was screened by a new, simple system, based on polyclonal rabbit antiserum raised against "GH secretagogue pharmacophore" of a known growth hormone secretagogue GHRP-6 (Hexarelin®) and the most promising GH secretagogue candidate was selected.

Key words: secretagogue, growth hormone, combinatorial chemistry.

Due to serious health problems, primarily undesirable side-effects, and the high cost of recombinant growth hormone (GH) treatment,[1] lot of attention has been paid during the past few years to the development of new GH secretagogues with a satisfactory oral bioavailability. The discovery of the first GH secretagogues, growth hormone releasing peptides (GHRPs),[2] led to the synthesis of different peptidyl and non-peptidyl compounds of this class. However, a methodological approach for the discovery of new GH secretagogues, that would involve the tools of combinatorial chemistry, is still lacking. This prompted us to develop an original methodological approach, involving the tools of combinatorial chemistry, which was further applied for the design and synthesis of a small library of peptide-like compounds, prospective groeth hormone secretagogues.

Since small peptides that contain α-methyl amino acid residues are thus, not susceptible to degradation by degestive enzymes, they might meet the structural and pharmacological demands for a suitable GH secretagogue candidate. Hence, a small library of tripeptoids on a soluble support was prepared and the most promising compound(s) were selected. The results are reported in the present work.

EXPERIMENTAL

1. The library synthesis was performed on a soluble polymeric carrier, polyethylene monomethyl ether 5000 (PEG 5000, Fluka Chemie AG, Switzerland), using seven building blocks: the tBoc[3a]-derivatives of 1. isonipecotic acid (ipe), 2. *S*-phenylalanine (phe), 3. glycine (gly), 5. *S*-glutamine (gln) and 6. *R,S*-α-methyl phenylanine (mphe), as well as the F-moc[3b] protected 4. aminoisobutyric acid (aib) and 7. *R,S*-α-(1-naphthylmethyl)-alanine (bnaf).[1]

The peptide bond formation (coupling) proceded in anhydrous dimethyl formamide (DMF; Scheme 1), with diisopropyl carbodiimide (DICI) as the coupling agent.[3c] Removal of the tBoc- and Fmoc-protective groups was accomplished in trifluoroacetic acid and 20% piperidine in DMF, respectively,[3d] as shown in Scheme 1. The intermediate and final products were precipitated and rinsed with anhydrous diethyl ether.

2. ELISA: A GHRP-6 sequence, attached to albumin egg *via* the pentapeptide linker (His-D-Trp-Ala-Trp-Ala-D-Phe-Lys-Gly-Ala-Asn-Ala-ovalbumin), was used as an antigen (10 μg/mL, 50 mM carbonate buffer, pH 9.6). Polyclonal rabbit antiserum, developed against GHRP-6 (Hexarelin®) and specific to the benzolactam secretagogue structure, was employed as the primary antibody source (dilution 1:5000 in phosphate-buffered saline, PBS), to which the products of the library were added as binding competitors (1.0 mM and 0.5 mM for the first and the second screening, respectively). Anti-rabbit IgG (Sigma, St. Luis, MO, U.S.A.), conjugated with alkaline phosphatase, was used as the secondary antibody source (dilution 1:500). Upon the introduction of the substrate (10 mg/mL *p*-nitrophenyl phosphate, 10% diethanolamine buffer, 1.0 mg/mL $MgCl_2$, pH 9.8) into the reaction mixture, the content of the reaction products was determined spectrophotometrically at 405 nm. The PBS used for rinsing and antisera dilutions was saturated with chicken egg albumin.[4]

RESULTS AND DISCUSSION

The library of potential GH secretagogues was designed using the "split and mix"[5] method, according to the PEG-phe-*x*-*y*, scheme, where *x* represents any building block, and *y* is any building block except block No. 7. The position at the C-terminus remained conserved. Employing this method of combinatorial chemistry, three subsequent libraries, based on the sreening of the preceding one, as illustrated in Figs. 1–3, were desgined.

Application of the reiterative deconvulsion method,[6] showed that PEG-phe-bnaf-gly (Fig. 4) was the most efficient inhibitor of the antigen-primary antibody interactions of all the compounds tested throughout this work, thus representing the most promising candidate as a suitable GH secretagogue.

It has been reported that some peptidyl structures with α-methyl groups act as GH secretagogues with increased potency.[7] This, by downsizing a secretagogue sequence from a hexapeptide (GHRP-6, Hexarelin®) to penta-, tetra- and tripeptides, new molecules with increased *in vitro* and *in vivo* activity were produced.[8] However, the combinatorial chemistry approach, applied throughout the present study, has not yet been reported in discovering new GH secretagogues. The PEG 5000 used here as a soluble carrier enabled a simple amplification of the "split-and-mix" method and allowed the application of classical peptide chemistry approaches

1 This numeration of the building blocks is used further in this paper.

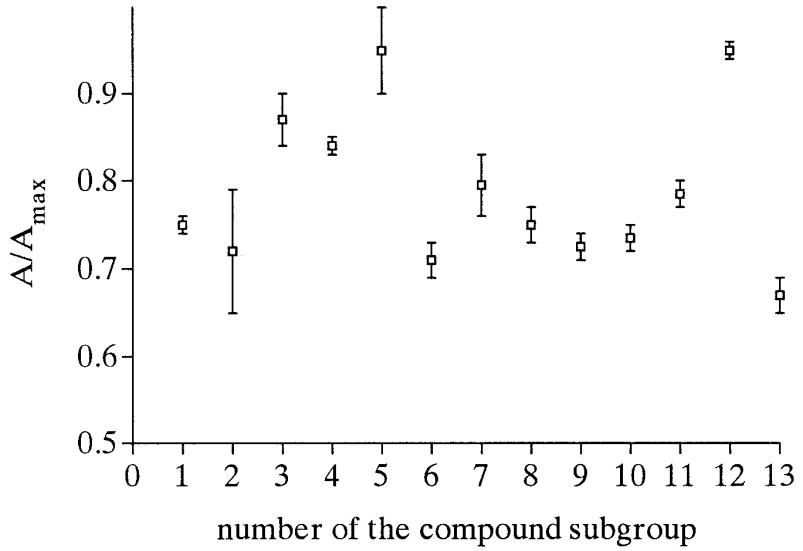

Fig. 1. Screening results of the first library: (□) Compound subgroups of the library PEG-phe-*x*-*y*: 1–PEG; *x*–(2–7) and (8–13)–ipe,phe,gly and aib,gln,mphe,bnaf, respectively; *y*–(2,8), (3,9), (4,10), (5,11), (6,12) and (7,13)– ipe, phe, gly, aib, gln and mphe, respectively.

without serious limitations. The efficiency of a new, simple sreening system, specific for the "GH secretagogue pharmacophore",[9] has also been demonstrated.

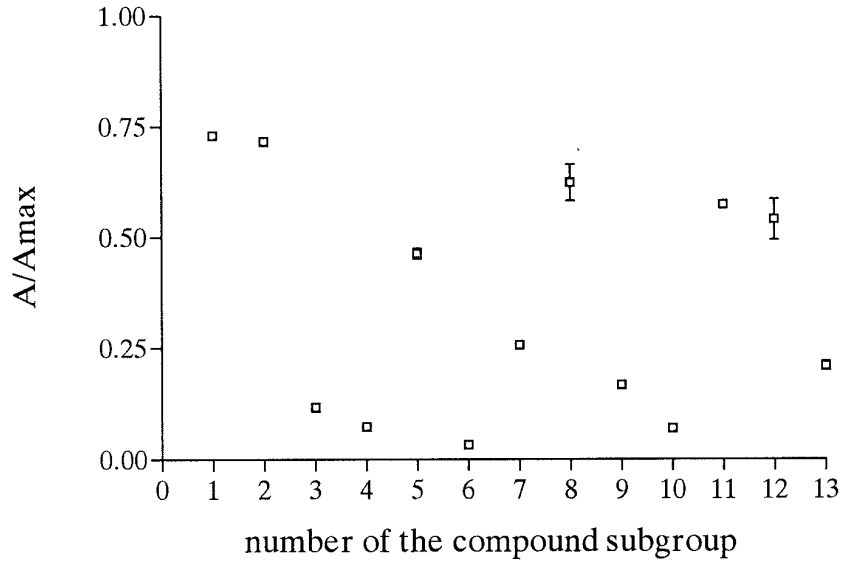

Fig. 2. Screening results of the second library: (□) Compound subgroups of the library PEG-phe-x_b-*y*: 1–PEG; x_b–(2–7) and (8–13), aib, mphe and gln, bnaf, respectively, *y* – (2,8), (3,9), (4,10), (5,11), (6,12) and (7,13) – ipe, phe, gly, aib, gln and mphe, respectively.

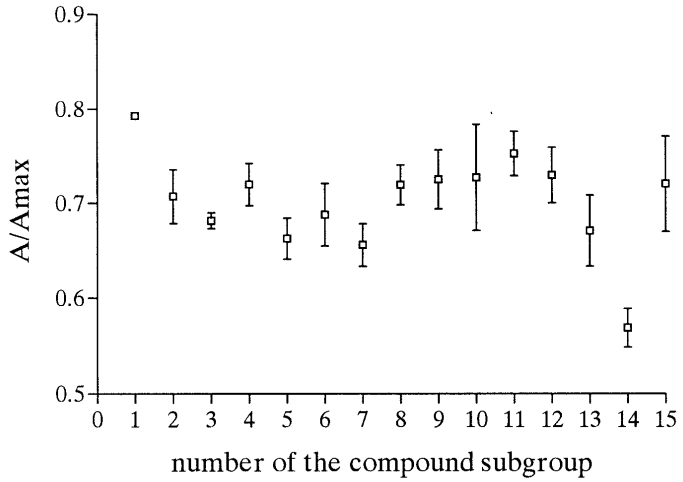

Fig. 3. Screening resulgs of the third library: (□) Compound subgroups of the library PEG-phe-x_c-y; 1–PEG; x_c – (2–5), (6–8), (9–12) and (13–15): aib, gln, mphe and bnaf, respectively, y – (2,6,9,13), (3,7,10,14), (4,8,11,15) and (5,12) – phe, gly, gln and mphe, respectively.

Screening of the first library showed that the most potent mixture contained aib, gln, mphe or bnaf at the second position in the sequence and mphe at position No. 3. Thus, the second library was made of two sublibraries (PEG-phe-x_a-y and PEG-phe-x_b-y, depicted in Figs. 2 and 3, respectively). The following sreening results gave three mixtures expressing similar "activity", and the subgroup PEG-phe-x_b-gly was chosen as the basis for the synthesis of library No. 3. (Mixtures of PEG-phe-x_a-gln and PEG-phe-x_a-gly were shown to possess similar inhibitory activity in the ELISA assay. However, our aim was to demonstrate a useful synthesis-screening strategy towards the discovery of new GH secretagogues. Therefore, selecting a good, single GH secretagogue candidate from these three mixtures would prove the strategy to be correct. The most active compound from the third library, PEG-phe-bnaf-gly (Fig.

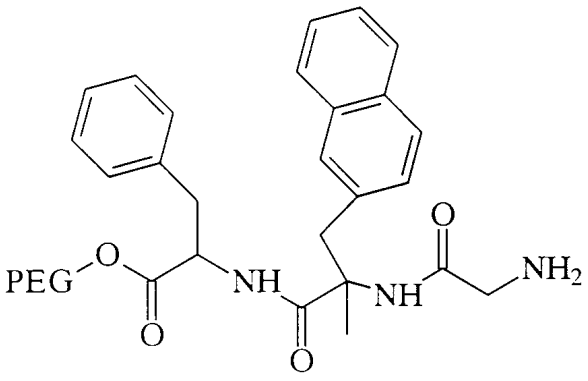

Fig. 4. Structure of the most active library compound and potential GH secretagogue.

4), shares structural features with both peptidyl and non-peptidyl GH secretagogues, represented by a hydrophobic middle region, *R*-configuration and a charged terminus of the molecule are essential for biological activity.[10,11] Since the bnaf component is racemic, it could be assumed that upon its resolution to enantiomers, the compound PEG-phe-*R*-bnaf-gly would be twice as active. This hypothesis is supported by the properties of the screening system developed against the GHRP-6 sequence, which recognizes benzolactam GH secretagogues and is able to reveal the difference between benzolactam *R*- (GH secretagogue active) and *S*- (GH secretagogue inactive) enantiomers.[9] The real significance of this potential GH secretagogue and this screening system should be further evaluated by binding and functional assays.

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И З В О Д

СИНТЕНА МАЛЕ БИБЛИОТЕКЕ ПОТЕНЦИЈАЛНИХ СЕКРЕТАГОГА ХОРМОНА РАСТА

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Применом поступака комбинационе хемије развијен је оригиналан методолошки приступ који је примењен за планирање и синтезу мале библиотеке једињења сличних пептидима, могућим секретагозима хормона раста. За те врсте је коришћено седам градивних блокова аминокисеилна заштићених везивањем за tBoc и Fmoc. На овај начин је добијена мала библиотека трипептоида на полиетилен гликол монометил етру (PEG 5000) као солубилном носачу. Ова библиотека је претраживана помоћу новог, једноставног система заснованог на поликлонском зечјем антисеруму развијеном против "фармакофоре" познатог секретагога хормона раста GHRP-6 (Hexarelin®) и издвојено је најактивније једињење које представља потенцијални секретагог хормона раста.

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