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Synthesis, biological activity and isomerism of guanylate cyclase C-activating peptides guanylin and uroguanylin

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Recently, the peptides guanylin and uroguanylin were identified as endogenous ligands of the membrane-bound guanylate cyclase C (GC-C) that is mainly expressed in the intestinal epithelium. In the present study, bioactive guanylin and uroguanylin have been prepared by solid-phase methodology using Fmoc/HBTU chemistry. The two disulfide bonds with relative 1/3 and 2/4 connectivity have been introduced selectively by air oxidation of thiol groups and iodine treatment of Cys(Acm) residues. Using this strategy, several sequential derivatives were prepared. Temperature-dependent HPLC characterization of the bioactive products revealed that guanylin-related peptides exist as a mixture of two compounds. The isoforms are interconverted within approximately 90 min, which prevents their separate characterization. This effect was not detected for uroguanylin-like peptides. Synthetic peptides were tested for their potential to activate GC-C in cultured human colon carcinoma cells (T84), known to express high levels of GC-C. The results obtained show that both disulfide bonds are necessary for GC-C activation. The presence of the amino-terminally neighboring residues of Cys104 for guanylin and Cys100 for uroguanylin has been found to be essential for GC-C stimulation. Unexpectedly, a hybrid peptide obtained from substitution of the central tripeptide AYA of guanylin by the tripeptide VNV of uroguanylin was not bioactive. © Munksgaard 1997.

Key words: cGMP; disulfide; guanylate cyclase C; guanylin; uroguanylin; peptide hormone; peptide synthesis; topological isomerism

Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; cGMP, cyclic 3′,5′-guanosine monophosphate; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; EDT, ethanedithol; ESMS, electrospray mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; GC-C, guanylate cyclase C; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAc, acetic acid; HOBt, *N*-hydroxybenzotriazole; Isc, short-circuit current; MeCN, acetonitrile; NMP, *N*-methylpyrrolidinone; NMR, nuclear magnetic resonance; PHB, *p*-alkoxybenzyl alcohol; TBME, *tert*-butylmethylether; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

Residue numbers refer to the circulating proguanylin-(22–115) (**7**) and cDNA-deduced uroguanylin precursor containing 112 amino acids (**11**) and are used throughout the text.

Part of this work has been presented in abstract form at the 4th International Symposium, Solid Phase Synthesis & Combinatorial Chemical Libraries, Edinburgh, Scotland [Adermann, K., Neitz, S., Marx, U., Rösch, P. & Forssmann, W.G. (1995) Abstract P1].

Guanylin and uroguanylin are novel mammalian peptide hormones that are ligands of intestinal receptor guanylate cyclase C (GC-C). GC-C activation increases the intracellular concentration of cyclic guanosine monophosphate (cGMP), which in turn modulates intestinal water and electrolyte secretion (1–5). The endocrine role of this new family of regulatory peptides with respect to epithelial ion transport and a functional link between intestine, kidney and other tissues are topics of current research in physiology. Guanylin, originally isolated as a peptide of 15 residues from rat jejunum (6), also circulates in human plasma as a larger hormone containing 94 amino acids (7). Uroguanylin was isolated from urine and represents a second activator of GC-C (8, 9). Recently, an amino-terminally elongated form of 24 amino acid residues was shown to represent a plasma-circulating form of uroguanylin (10, 11). It was also reported that prouroguanylin is present in plasma and urine in patients with chronic renal failure (12).

Both guanylin and uroguanylin share a significant sequence homology with a specific family of bacterial enterotoxins (Fig. 1). In particular, *Escherichia coli* heat-stable enterotoxin STa causing secretory diarrhea in mammals was shown to activate GC-C with a significantly higher efficacy than guanylin and uroguanylin (13, 14). It was predicted that the four cysteine residues of guanylin and uroguanylin form two disulfide bonds in a connectivity that is also contained in *E. coli* STa (15, 16). Synthesis of the three possible disulfide isomers of guanylin confirmed that only one of the several possible disulfide isomers acts as a GC-C activator and therefore is able to increase the intracellular cGMP level (2, 17). Similarly, uroguanylin with equivalent disulfide connectivity was shown to stimulate GC-C in a concentration-dependent manner (9, 10). It is generally accepted that the other two disulfide isomers of uroguanylin are biologically inactive.

Considering the molecular interaction of guanylin and uroguanylin with the extracellular domain of their receptor, the disulfide loops drastically reduce their conformational flexibility, resulting in a highly constrained three-dimensional structure. In this context, a unique feature of guanylin and uroguanylin is that both peptides exist in two distinct conformations. Although not separable by chromatographic methods, it was reported that, for synthetic guanylin-(103–115) and the tryptic fragment (94–115) obtained from recombinant proguanylin, two isomers are present in aqueous solution, as detected by NMR spectroscopy (18). In that study, it was concluded that one of the two forms adopts a conformation similar to that observed for *E. coli* enterotoxin STa (19). We have shown that guanylin-(99–115) also appears as a mixture of two compounds which are detectable by means of low-temperature HPLC (20). Very recently, it was reported that solid-phase synthesis of human uroguanylin-(97–112) yields two topological isomers which could be separated by HPLC (21). In another study, it was reported that only one of these isomers is bioactive and different native forms of uroguanylin have been identified using polyclonal antisera raised against the two conformational isomers of synthetic uroguanylin (12).

In this communication, we describe a general strategy that allows solid-phase synthesis of guanylin/uroguanylin-type peptides with regioselective introduction of disulfide bonds. Using this tool, several derivatives of guanylin and uroguanylin have been synthesized to gain insight into sequential and conformational requirements for the interaction between the peptide regulators and their receptor.

Guanylin (99-115)	EDPGTCEICAYAACTGC
Uroguanylin (97-112)	NDDCELCVNVACTGCL
Uroguanylin (89-112)	FKTLRTIANDDCELCVNVACTGCL
E. coli STa	NTFYCCELCCNPACTGCY

FIGURE 1

Primary structures of GC-C-activating peptides. Conserved residues are shaded.

EXPERIMENTAL PROCEDURES

Reagents for peptide synthesis. Fmoc amino acids (L-configuration) were purchased from Orpegen (Heidelberg, Germany) and PerSeptive Biosystems (Wiesbaden, Germany). Protective groups were: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Acm), Cys(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu), Tyr(tBu). Syntheses were carried out using TentaGel resins (Rapp Polymere, Tübingen, Germany). HBTU was obtained from Perkin-Elmer/ABI (Weiterstadt, Germany), TBTU was from Peboc (Llangefni, Wales). NMP was purchased from Merck (Darmstadt, Germany). Other reagents and solvents were of analytical or higher grade and obtained from Merck or Fluka (Neu-Ulm, Germany). *E. coli* heat-stable enterotoxin STa was from Sigma (Deisenhofen, Germany).

Solid-phase peptide synthesis. Peptide assemblies were carried out in NMP at a scale of 0.1 mmol using a preloaded Fmoc-Cys(Trt)-TentaGel S Trt or S PHB resin (0.23 mmol/g) for guanylin-related peptides and Fmoc-Leu-TentaGel S Trt (0.19 mmol/g) for uroguanylin-related peptides. For the synthesis of guanylin peptides, Cys104 and Cys112 were Acm-protected, whereas Cys107 and Cys115 were Trt-protected. Uroguanylin was synthesized with Trt protection for cysteines in positions 103 and 111, and Acm protection at Cys100 and Cys108. As a standard procedure, all peptides were synthesized on a 433A peptide synthesizer (Perkin-Elmer/ABI) with conditional conductivity monitoring of Fmoc deprotections. In addition, guanylin-(99–115) and uroguanylin-(89–112) were synthesized manually on preloaded TentaGel S PHB resins (2 mmol) using double coupling cycles for each residue (10 mmol Fmoc amino acid, 9.5 mmol TBTU, 4 mmol HOBt and 20 mmol DIEA). Acylations were performed for 2 h and monitored by ninhydrin assay (22). Fmoc groups were cleaved with 20% piperidine in NMP for 20 min. After completion of syntheses, resins were washed with DCM and dried under vacuum. For resin cleavage and deprotection, the dry peptidyl resins were treated at room temperature with a fresh mixture of TFA/EDT/H_2O (94:3:3, 20 mL/g) for 1.5–2 h. Subsequently, the peptide-containing cleavage cocktail was filtered and slowly dropped into chilled TBME (100 mL/20 mL TFA). Precipitated crude peptides were separated by centrifugation, washed twice with cold TBME and dried under vacuum without lyophilization.

Disulfide formation and purification. To introduce the first disulfide bond by air oxidation, the crude peptide was dissolved in water (0.5–1 mg/mL). pH was ad-

justed between 7.8 and 8.5 with diluted NH_3. The solutions were stirred until no free thiol was detectable by Ellman's reagent (23) and C18-HPLC monitoring. Mixtures were acidified with TFA (pH 2.5), loaded directly onto a preparative C18-HPLC column and purified (Vydac C18, Hesperia, CA, 47 × 300 mm, 300 Å, 15–20 µm, buffer A; 0.1% TFA, buffer B: 0.1% TFA in MeCN/water, 80:20, gradient 0–100% B in 60 min, flow 50 mL/min, UV detection at 230 nm). Fractions containing the pure monocyclic intermediate as detected by ESMS and C18-HPLC were collected and lyophilized. Cleavage of Acm groups and introduction of the second disulfide bond were carried out by dissolving the purified monocyclic Acm_2-peptides in *deairized* HOAc/0.1 M HCl (4:1, pH 2, 1 mg/mL). A solution of iodine (10–20 eq, 0.05 M) in HOAc was added and after 30-60 min the excess iodine was reduced by the addition of fresh 0.1 M sodium thiosulfate, resulting in a colorless solution. After dilution with water (2 volumes), the bicyclic products were obtained from preparative C18-HPLC (Vydac C18, 20 × 250 mm, 300 Å, 10 µm, buffer A: 0.1% TFA; buffer B: 0.1% TFA in MeCN/water 80:20, gradient 0–100% B in 60 min, flow 8 mL/min, 230 nm). Purification was performed at 50 °C (guanylin peptides) or room temperature (uroguanylin peptides). In the case of insufficient purity, HPLC purification of bicyclic products was repeated.

Analytical methods. Analytical HPLC was performed on a Vydac C18 column (5 µm, 300 Å, 4.6 × 250 mm, buffer A: 0.06% TFA; buffer B: 0.05% TFA in 80% MeCN, flow rate 0.8 mL/min, 215 nm). Temperature-dependent HPLC characterization of purified peptides was carried out using an adjustable column oven with a Nucleosil C18 PPN column (2 × 250 mm, 5 µm, 100 Å, flow rate 0.2 mL/min, 215 nm, Macherey & Nagel, Düren, Germany). Mass spectrometry was performed using a triple-stage quadrupole mass spectrometer (Sciex API III, Perkin-Elmer). Peptide samples were dissolved in MeCN/water (1:1) containing 0.2% AcOH to a concentration of 0.1 mg/mL and infused into the ion spray using a syringe pump at a flow rate of 5 µL/min. The ion spray interface was set to a positive potential of 5 kV. Amino acid analysis was carried out on a Aminoquant 1090L analyzer (Hewlett-Packard, Waldbronn, Germany). Sequence analysis was performed on a 494A protein sequencer (Perkin-Elmer). Two-dimensional NMR spectroscopy (NOESY, Clean-TOCSY, DQF-COSY) was performed on a Bruker AMX600 spectrometer at 284 K using standard methods (24, 25). Synthetic peptides were dissolved in H_2O/D_2O (9:1) at a concentration of 5–10 mg/mL.

T84 cell cGMP bioassay. Determination of the GC-C-stimulating activity of synthetic peptides was evaluated as described elsewhere in detail (1, 7, 10). Cultured human colon carcinoma (T84) cells were incubated with synthetic peptides for 60 min in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1 mM, Sigma). The peptides were tested in concentrations of 10^{-9} to 10^{-6} M. The given concentrations refer to the net peptide content specified by amino acid analysis. Effects on intracellular cGMP level were compared with those of *E. coli* enterotoxin STa. cGMP was measured using a specific radioimmunoassay (26).

Ion transport (Ussing chamber experiments). Female Wistar rats maintained under standard temperature, light conditions and with free access to water and food were used. For the assessment of active electrogenic ion transport, specimens of proximal colon were dissected and serosal and muscular layers separated. Sheets of isolated mucosa were mounted in Ussing chambers (27) with an exposed surface area of 1 cm^2 and automatically voltage-clamped as described previously (3, 4). Short-circuit current (Isc) was recorded continuously. After an equilibrium period of 30 min, cumulative concentrations of synthetic peptides were added to the luminal side of the colonic mucosa.

RESULTS AND DISCUSSION

Synthesis

In agreement with earlier observations (2, 21) and in contrast to other peptides like endothelin (28) and low molecular weight toxins (29), oxidative folding of tetrathiol-containing precursors of guanylin and uroguanylin did not result in bioactive products that increase intracellular cGMP concentration. The reduced peptides had a poor solubility in aqueous buffers. Further attempts to synthesize guanylin with different sets of orthogonal side-chain protective groups for the cysteine residues like acetamidomethyl (Acm), *tert*-butyl (tBu), *tert*-butylthio (StBu), *p*-methoxybenzyl (Mob) or allyloxycarbonylaminomethyl (Allocam) resulted in products that were only slightly soluble in buffers required for disulfide formation. To improve solubility, guanylin was initially synthesized with additional amino-terminal Glu-Asp residues which do not influence the bioactivity. Guanylin and uroguanylin were synthesized using Acm and Trt protection for cysteine residues with Cys(Trt) at the C-terminal position. Detectable by conductivity monitoring, qualitative ninhydrin assay or HPLC/ESMS of intermediates, guanylin and uroguanylin represent difficult peptides. Starting from Ala110 (guanylin) and Val106 (uroguanylin), acylations and cleavage of Fmoc groups tended to proceed slowly. For the manual synthesis of larger amounts of peptides, acylations were carried out for 2 h whereas Fmoc peptidyl resins were treated for 20 min with piperidine. Exchanging the pairs of cysteine protective groups resulted in a product of slightly less purity.

The first loop between the unprotected cysteines of

all synthetic compounds was introduced by air oxidation at pH 8. The proceeding oxidation process could be monitored easily by Ellman's test (23), HPLC comparison of thiol peptides and monocyclic products or ESMS analysis after iodoacetamide treatment (30) (Fig. 2). Depending on the sequence, a period of 12–72 h was necessary for this step. For the subsequent work-up of monocyclic Acm_2-peptides, it was essential to acidify the mixture to pH 2.5 (HCl or TFA). As detected by MS and HPLC, direct lyophilization or purification of a pH 8 solution of monocyclic peptides resulted in products with a poor solubility, possibly caused by further oxidation of cysteines. To obtain final products of a high purity, additional HPLC purifications of the monocyclic Acm_2-peptides were performed. Cleavage of Acm groups with subsequent formation of the second disulfide with excess iodine at pH 2 (HCl) was a suitable method with suppression of disulfide rearrangement (31). The absence of HCl led to unsatisfactory products. Different solvents like mixtures of water, acetic acid and methanol did not affect iodine treatment. Alternative attempts at disulfide formation using on-resin procedures and the solvent-dependent selectivity of iodine treatment of free thiols and Acm-protected cysteine residues failed (32); dimerization was a significant side effect. Overall yields were between 2 and 8% because of the repeated HPLC purifications of mono- and bicyclic peptides. With guanylin-(99–115) and uroguanylin-(89–112) as examples, Fig. 2 shows analytical HPLC of intermediate compounds and final products which were unambiguously characterized by ESMS. Using this general synthetic procedure, guanylin and uroguanylin as well as several derivatives have been prepared and characterized (Table 1). As recently comprehensively reviewed (31, 33), our results of the synthesis of guanylin and uroguanylin are another indication that suitable reaction conditions for each single peptide containing multiple disulfide bonds must be worked out accurately.

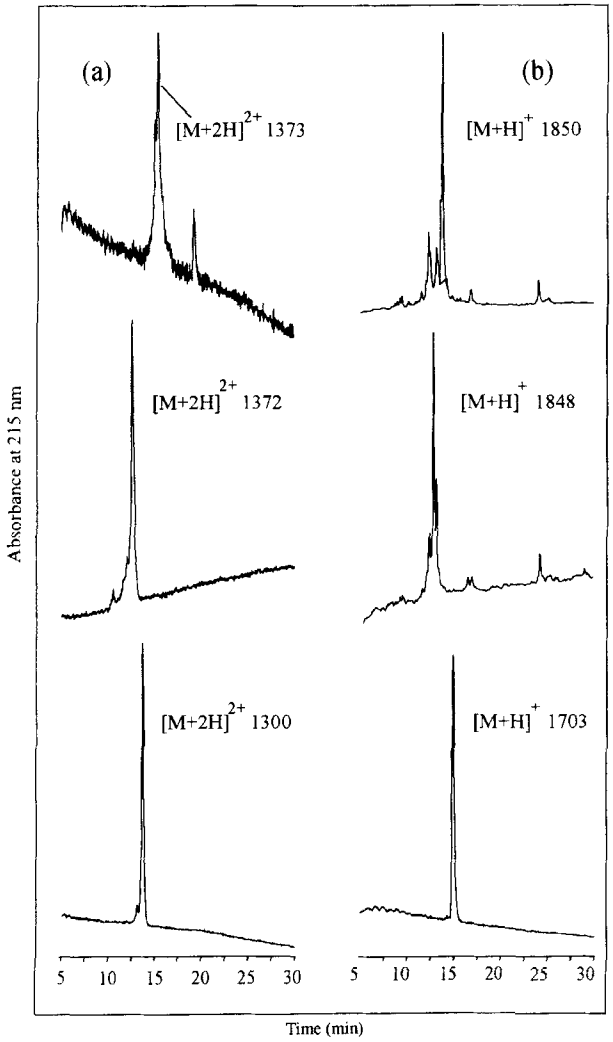

FIGURE 2

HPLC characterization of linear, monocyclic and purified peptides. (a) human uroguanylin-(89–112) FKTLRTIANDDCELCVNVACTGCL; (b) human guanylin-(99–115), EDPGTCEICAYAACTGC. Top traces, linear peptides after TFA cleavage; middle traces, monocyclic Acm_2-peptides; bottom traces, bicyclic, purified products. HPLC gradient (Vydac C18): linear and monocyclic peptides, 20–70% buffer B in 30 min at room temperature; bicyclic peptide, 10–50% buffer B in 40 min at 40 °C. Masses given were determined by ESMS.

Unlike other polycyclic peptides with multiple cystines within a short sequence segment, low-temperature HPLC analysis of purified guanylin-(99–115) (**4**) showed two components in a ratio of about 1:1. ESMS analysis resulted in only one signal with the expected molecular weight of 1703 Da. Reverse-phase HPLC with a stepwise increase of column temperature led to one sharp absorption at high temperature. *Cis/trans* isomerism of the Asp100-Pro101 amide bond was excluded as the reason for this observation by temperature-dependent HPLC analysis of guanylin-(101–115) (**3**) with an amino-terminal Pro residue. Shown in Fig. 3, a and b, peptides **3** and **4** exhibit the identical HPLC characteristic at different temperatures. A corresponding temperature effect was observed with guanylin-(104–115) (**1**), [desThr103]guanylin-(99–115) (**7**) and compounds **12** and **13**. In contrast, guanylin-(103–115) (**2**) appears as a single HPLC peak. This is consistent with an earlier report (18). Our preliminary NMR experiments with guanylin-(99–115) (**4**) confirmed the presence of two distinct compounds in a ratio of about 1:1 (20). The guanylin/uroguanylin hybrid **9** had a major HPLC peak, but two-dimensional NMR spectroscopy revealed the presence of two components in equivalent amounts (data not shown). As expected, monocyclic $Cys(Acm)_2$ intermediates **10**, **11** and **18** were homogeneous during HPLC. A reversed in-

TABLE I

Analytical data of synthetic peptides

No.	Sequence	Amino acid composition
1	CEICAYAACTGC	Ala 3.06 Ile 0.98 Glx 0.68 Gly 1.13 Thr 0.84 Tyr 0.75
2	TCEICAYAACTGC	Ala 3.09 Ile 0.95 Glx 0.59 Gly 1.02 Thr 1.60 Tyr 0.88
3	PGTCEICAYAACTGC	Ala 3.31 Ile 1.00 Glx 0.63 Gly 0.99 Thr 1.56 Tyr 0.94 Pro 0.92
4	EDPGTCEICAYAACTGC	Ala 3.02 Ile 0.94 Asx 0.97 Glx 1.56 Gly 2.01 Thr 1.61 Tyr 0.84 Pro 1.08
5[a]	EDPGTCEICAYAACTGC	Ala 3.11 Ile 0.95 Asx 0.89 Glx 1.37 Gly 2.23 Thr 1.73 Tyr 0.86 Pro 1.12
6	EDPGTCICAYAACTGC	Ala 3.22 Ile 0.93 Asx 0.90 Glx 0.72 Gly 1.98 Thr 1.63 Tyr 0.79 Pro 0.98
7	EDPGCEICAYAACTGC	Ala 3.06 Ile 0.94 Asx 0.66 Glx 1.12 Gly 2.02 Thr 0.90 Tyr 0.81 Pro 1.01
8	EDPGTCEICAGGACTGC	Ala 2.02 Ils 0.97 Asx 0.81 Glx 1.31 Gly 4.06 Thr 1.72 Pro 1.06
9	EDPGTCEICVNVACTGC	Ala 1.01 Ile 0.97 Asx 1.83 Glx 1.42 Gly 2.02 Thr 1.77 Pro 1.02 Val 1.93
10[b]	EDPGTC(Acm)EICAYAAC(Acm)TGC	n.d.
11[c]	EDPGTCEIC(Acm)AYAACTGC(Acm)	n.d.
12[d]	PSTCEICAYAACAGC	Ala 4.12 Ile 0.93 Glx 0.63 Gly 1.15 Thr 0.78 Tyr 0.88 Pro 1.04 Ser 0.45
13	PNTCEICAYAACTGC	Ala 3.08 Ile 0.98 Asx 0.78 Glx 0.65 Gly 1.03 Thr 1.70 Tyr 0.82 Pro 1.02
14	NDDCELCVNVACTGCL	Ala 1.04 Val 1.89 Leu 2.04 Asx 3.84 Glx 0.72 Gly 1.19 Thr 0.93
15	FKTLRTIANDDCELCVNVACTGCL	Ala 2.12 Arg 1.04 Val 1.94 Phe 0.98 Ile 0.98 Leu 3.05 Asx 3.95 Glx 0.71 Gly 1.11 Thr 2
16	FKTLRTIANDCELCVNVACTGCL	Ala 2.08 Arg 1.01 Val 1.94 Phe 1.00 Ile 0.93 Leu 3.10 Asx 3.09 Glx 0.71 Gly 1.23 Thr 2
17	FKTLRTIANDDCLCVNVACTGCL	Ala 2.07 Arg 0.99 Val 2.02 Phe 0.97 Ile 0.98 Lei 3.05 Asx 4.06 Gly 1.17 Thr 2.21 Lys 0.
18[e]	FKTLRTIANDDC(Acm)ELCVNVAC(Acm)TGCL	n.d.
19[f]	LQALRTMDNDECELCVNIACTGC	Ala 2.13 Arg 0.99 Val 0.96 Met 0.95 Ile 1.00 Leu 3.08 Asx 3.99 Glx 2.35 Gly 1.20 Thr 1
20[g]	(BiotinAhx)FKTLRTIANDDCELCVNVACTGCL	n.d.

[a]Reversed order of disulfide bond formation using Cys(Acm)107/115 and Cys(Trt)104/112.
[b]Monocyclic Acm_2-intermediates.
[c]Monocyclic Acm_2-intermediates.
[d]Sequences from porcine and guinea pig guanylin obtained by translation of the corresponding mRNA (EMBL database, Accession nos. Z73607 and Z7
[e]Monocyclic Acm_2-intermediates.
[f]Sequence from guinea pig uroguanylin obtained by translation of the corresponding mRNA (EMBL database, Accession no. Z74738).
[g]BiotinAhx, *N*-(Biotinyl-6-aminocaproyl).
[h]$[M+H]^+$.
[i]$[M+2H]^{2+}$.
n.d., not determined.

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