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The unique C-mannosylated hypertrehalosemic hormone of *Carausius morosus*: Identity, release, and biological activity

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Abstract

Previous studies had shown that the corpora cardiaca (CC) of the Indian stick insect, *Carausius morosus*, synthesizes two hypertrehalosemic hormones (HrTHs)—decapeptides which differ in the way that the chromatographically less-hydrophobic form, code-named Carmo-HrTH-I, is modified by an unique C-mannosylated tryptophan residue at position 8. The availability of milligram amounts of this modified peptide in synthetic form now makes it possible to study physico-chemical and physiological properties. This study revealed that the synthetic peptide co-elutes with the natural peptide from the CC chromatographically, is heat stable for at least 30 min at 100°C, and causes hyperlipemia in acceptor locusts (a heterologous bioassay) and hypertrehalosemia in ligated stick insects (conspecific bioassay). In vitro incubation of Carmo-HrTH-I together with stick insect hemolymph (a natural source of peptidases) demonstrated clearly via chromatographic separation that the C-mannosylated Trp bond is stable and is not broken down to Carmo-HrTH-II (the more-hydrophobic decapeptide with an unmodified Trp residue). This notwithstanding, breakdown of Carmo-HrTH-I did take place, and the half-life of the compound was calculated as about

5 min. Finally, the natural peptide is releasable when CC are treated in vitro with a depolarizing saline (high potassium concentration) suggesting its role as true HrTHs in the stick insect. In conclusion, the results indicate that Carmo-HrTH-I which is synthesized in the CC is released into the hemolymph, binds to a HrTH receptor in the fat body, activates the carbohydrate metabolism pathway and is quickly inactivated in the hemolymph by (an) as yet unknown peptidase(s).

KEYWORDS

adipokinetic hormone family, *Carausius morosus*, hyperlipemic and hypertrehalosemic bioassay, Indian stick insect, neuropeptide release in vitro, peptide half-life, peptide inactivation, RP-HPLC, Trp C-mannosylated peptide

1 | INTRODUCTION

Peptides of the adipokinetic hormone (AKH) family in insects are synthesized and stored in neurosecretory cells of the corpora cardiaca (CC), a well-known neurohemal organ, from where the neuropeptides are released into the hemolymph. In most insect species the AKHs are the major abundant neuropeptides; for example, more than 3.4 nmoles are stored in the CC of the Lubber grasshopper, *Romalea microptera* (Spring & Gäde, 1991). To date, almost 100 structurally related AKHs have been fully chemically identified in insects (Gäde, 2009; Gäde et al., 2016, 2019, 2020; Marco et al., 2020). Key signature features of the octa-, nona- or decapeptide AKHs are the blocked termini (a pyroglutamate [pGlu] residue at the N-terminus and a carboxyamide at the C-terminus), aliphatic amino acids such as leucine (Leu), isoleucine (Ile) and valine (Val) or phenylalanine (Phe) at position 2, asparagine (Asn) or threonine (Thr) as residue 3, Phe or tyrosine (Tyr) at position 4, Thr or serine (Ser) as residue 6, tryptophan (Trp) at position 8 and glycine (Gly) at position 9, whereas positions 5, 7 and 10 can be any of a variety of amino acids (Marco & Gäde, 2020). Once the AKH is released into the hemolymph it is transported through the body and binds to a G protein-coupled receptor (GPCR) on the fat body to activate finally, either a lipase or a phosphorylase to respectively breakdown stored triacylglycerides (TAG) or glycogen via different pathways (Gäde & Auerswald, 2003). Thus, the main function of AKHs is regulation of energy metabolism and it plays a major role during strenuous muscular activity, such as flight, which is an entirely aerobic process in insects.

However, AKHs also occur in flightless species. One example is the Indian stick insect, *Carausius morosus*, that uses thanatosis (playing dead) to avoid detection, instead of investing in physical energy to flee from predators. More than 40 years ago it was demonstrated that a *C. morosus* CC extract had adipokinetic and hypertrehalosemic activity, respectively, when injected heterologously into migratory locusts and American cockroaches (Gäde, 1979); the active factor is entirely found in the CC, not in other nervous tissue, and it is already present in CC of 2nd instar stick insects albeit 30 times less in quantity than in CCs of adults (Gäde, 1980). The stick insect CC extract was able to activate the fat body phosphorylase of *Locusta migratoria*; purification of a CC extract on Sephadex G-25 resulted in one peak with adipokinetic and phosphorylase-activating activity in locusts and further purification of this peak on Sephadex LH-20 resolved two peaks which had both activities (Gäde, 1981). Interestingly, the *C. morosus* CC

extract is not active in mobilizing energy metabolites (lipids or carbohydrates) in adult stick insects (Gäde, 1979) unless the recipient *C. morosus* is ligated and the hemolymph flow is impaired between head and rest of the body (Gäde and Lohr, 1982). Only under such a ligation condition is a hypertrehalosemic response measured in *C. morosus* itself: the highest increase in the concentration of hemolymph carbohydrates upon injection of a conspecific CC extract was achieved in 6th instar larvae and the two peaks derived from Sephadex separations of a crude *C. morosus* CC extract were also both active in hypertrehalosemia in ligated stick insects (Gäde & Lohr, 1982). With the faster and more efficient method of reversed-phase high-performance liquid chromatography (RP-HPLC), one smaller peak and one much larger later-eluting absorbance peak were purified from a CC extract of *C. morosus*, with adipokinetic activity shown in migratory locusts (Gäde, 1984) and hypertrehalosemic activity shown when tested in ligated stick insects (Gäde, 1985). Sufficient material of the second (more-hydrophobic) peak was collected, and an amino acid composition proposed for a nonapeptide (Gäde, 1985). This was incorrect as structural studies using fast atom bombardment mass spectrometry (FABMS) showed: the mass spectrum clearly assigned a decapeptide with the sequence pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide for the hydrophobic peptide of *C. morosus*, which is today code-named Carmo-HrTH-II (Gäde & Rinehart, 1987). An antiserum to Carmo-HrTH-II was prepared by coupling the nonapeptide (without the N-terminal pGlu) to thyroglobulin to produce a larger protein which has better immunogenic properties (Clottens et al., 1989). Immunohistochemistry resulted in a clear demonstration of immunoreactive cells only in the glandular part of the CC of the stick insect. The less-hydrophobic early-eluting peak, named Carmo-HrTH-I, gave more problems and surprises during identification (Gäde et al., 1992). First, the purified fraction gave an amino acid composition that was identical to that of Carmo-HrTH-II. Second, after enzymatic digestion of the pGlu blockage of the N-terminus with an oxoprolyl peptidase (=pyroglutamyl-peptidase), the peptide was subjected to automated Edman degradation, and the sequence information was identical to Carmo-HrTH-II, except at position 8 the analysis yielded no result where the Trp residue was expected. The two peptides could not possibly be identical anyway, despite the identical amino acid composition, because the two peptides were separated by hydrophobicity: compound I eluted a few minutes earlier, thus had to be more polar. Third, low resolution FABMS detected a $[M + H]^+$ ion at *m/z* 1308, and tandem FABMS on this ion gave daughter ions which assigned the peptide as identical to compound II but for the Trp residue which had a 162 amu higher mass suggesting the addition of a hexose. Glycosylation was only known as N-glycosylation (at Asn residue) or as O-glycosylation (at Ser or Thr residues) but here in the stick insect peptide Carmo-HrTH-I the Trp residue was modified, and it was speculated that the hexose may be attached to the nitrogen of the indole ring of Trp (Gäde et al., 1992). After nearly two decades of rearing more than 2000 stick insects to dissect CCs, extract and purify sufficient quantities of Carmo-HrTH-I peptide via RP-HPLC, nuclear magnetic resonance spectroscopy could be employed to solve the structural enigma around the glycosylated Trp residue (Munte et al., 2008). The Trp residue of Carmo-HrTH-I has an α-mannopyranose which is C-bonded. Trp C-mannosylation is the only known form of C-glycosylation and is quite rare; it was first structurally fully characterized in human RNase U_s (De Beer et al., 1995; Hofsteenge et al., 1994). The biological functions of such Trp-modifications in peptides and proteins is relatively poorly understood and that is—at least, partially—due to the methodological difficulties to synthesize such compounds. Recently, a new method was developed which made it possible, for the first time, to perform an automated solid-phase peptide synthesis of a Trp C-mannosylated peptide (Mao et al., 2021). One of the peptides that was synthesized was Carmo-HrTH-I. The availability of this peptide in its synthetic form (about 4 decades after its presence was first noted) allows us to finally investigate a number of questions we could not tackle before. Thus, in the present work the synthetic and natural peptide are chromatographically compared, the biological activity of the synthetic compound is established in two in vivo bioassay systems, the intactness of the C-bond after heat treatment and after incubation in vitro with hemolymph is shown, and the potential of the release of this peptide from the CC during depolarization is demonstrated in vitro. A rough calculation of the half-life of the hormone in the hemolymph is given as well. All these data give us a better insight into the function of this unusually modified AKH peptide in the stick insect.

2 | MATERIALS AND METHODS

2.1 | Insects

Sixth instar nymphs and young adult stick insects, *C. morosus*, were gifts from Prof. V. Dürr (University of Bielefeld) and Prof. A. Büschgens (University of Cologne, Germany). They were kept under crowded conditions at about 25 ± 2°C and a 12 h day-night cycle. They were fed ad libitum with fresh bramble leaves replaced every second day.

Young adults of both sexes of the migratory locust, *L. migratoria*, were purchased from a local pet shop in Osnabrück (Germany) and kept crowded but separated by sex at about 28 ± 2°C and a 12 h day-night cycle. Locusts were fed daily ad libitum with fresh grass and dandelion leaves supplemented with rolled oats and wheat bran.

2.2 | Biological assays

Adipokinetic activity was measured by injecting the test material into resting adult acceptor locusts of both sexes similar to early reports (Gäde, 1980). In brief, a 1 µL aliquot of hemolymph was taken from the base of the hindleg through the arthrodial membrane with a glass capillary and immediately blown into 100 µL of concentrated sulfuric acid. Subsequently the locust was injected with 10 µL of the test solution through the lower abdominal membrane and rested for 90 min whereupon a second 1 µL hemolymph sample was taken.

Hypertrehalosemic activity was assayed by injecting the test material into ligated, resting acceptor stick insects similar to earlier reports (Gäde & Lohr, 1982). In brief, late 6th instar stick insects or young adults (up to 10 days old) were ligated with woolen thread just behind the head in the neck region and kept resting for 2 h before experimentation. A 1 µL aliquot of hemolymph was taken at the base of the hindleg and blown into sulfuric acid. The test solution (10 µL) was then injected through the abdominal membrane into the stick insect. After a rest period of 90 min, a second 1 µL hemolymph sample was taken from the animal.

The colorimetric measurements of total lipids and carbohydrates were done according to Zöllner and Kirsch (1962) and Spik and Montreuil (1964) as modified by Holwerda et al. (1977). Two biological repeats were performed, with distilled water and synthetic Carmo-HrTH-I as controls. The difference in metabolite concentration pre- and postinjection for each test substance was tested for statistical relevance via a Student's paired *t*-test using Excel.

2.3 | Dissection of CC and separation of peptides by RP-HPLC

CC were dissected from the head of adult stick insects using a stereomicroscope at 20-fold magnification. Dissected CCs were placed into 80% vol/vol methanol, and peptide material was extracted by sonification and centrifugation; the supernatant was concentrated and dried by vacuum-centrifugation (see Gäde et al., 1984). Such crude extracts were taken up in 15% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and applied to a Nucleosil C-18 column (dimensions: 4 mm i.d. × 250 mm) with 5 µm particle size material equipped with a 20 mm long guard column containing the same material. Column temperature was maintained at 30°C. A Gilson HPLC separation system was used. The aqueous solvent A was 0.11% TFA. Solvent B was 0.10% TFA in 60% acetonitrile (HPLC grade, UV cut-off below 190 nm). The solvents were applied as a linear gradient from 43% to 53% B in 20 min at a flow rate of 1 mL/min. The eluent was monitored with a fluorimeter (Jasco) at 276 nm (excitation)/350 nm (emission) targeting the signature amino acid tryptophan which occurs at position 8 in all members of the AKH family. The very shallow gradient

(almost isocratic) plus fluorometry detection is paramount for a sensitive peak detection, thus, the same set-up was used with saline samples (Section 2.4 AKH release), as well as testing the stability of synthetic peptides (Section 2.5).

Natural peptides from *C. morosus* were collected as RP-HPLC fractions, dried in a vacuum concentrator and redissolved in water for use in biological assays.

2.4 | Release of AKHs from CC

The ability of the *C. morosus* CC to release all forms of bioactive AKH material was investigated in vitro by incubating intact glands of adult stick insects in saline with low and high concentrations of potassium to stimulate peptide release as outlined previously (Gäde, 1989) and then subjecting the CC-exposed saline solutions to RP-HPLC following the incubation steps. Briefly, CCs from 10 stick insects were pooled in an Eppendorf tube containing 50 µL low-K^+ saline and left to incubate for 30 min at 25°C in a shaker at 300 rpm; the saline was carefully removed from the tube so as not to damage the CCs and dried for HPLC in a speedvac. Immediately after removal of the low-K^+ saline from the incubation tube, high-K^+ saline (50 µL) was added to the CCs and incubated in the same way; the saline was carefully removed from the tube and dried for HPLC. Two biological repeats were performed. See Section 2.3 for HPLC conditions.

2.5 | Stability of the Trp C-bond and determination of half-life of the hormone

2.5.1 | Stability of the Trp C-bond to high temperature

A known amount (50 pmol) of synthetic Carmo-HrTH-I (the Trp-mannosylated compound) and synthetic Carmo-HrTH-II (the decapeptide with an unmodified Trp residue) were separately incubated for various time intervals (15–30 min) in Eppendorf tubes at 100°C and subsequently run on HPLC to monitor peak height (see Section 2.3 for HPLC conditions).

2.5.2 | Stability of the Trp C-bond during incubation with hemolymph; determination of half-life of the hormone

Freshly drawn hemolymph (10 µL) from an adult stick insect was added to an Eppendorf tube containing a 10 µL aliquot of synthetic Carmo-HrTH-I (amounting to 100 pmol), after a brief vortex to mix the solutions, the tube was incubated at RT (room temperature) for a timed period of 0–15 min before addition of 30 µL of 30% acetonitrile with 0.1% TFA to stop endogenous enzyme activity in the hemolymph, after which the tube was vortexed again followed by centrifugation (5 min at 21,000*g*). The resulting supernatant was injected to the HPLC system (Section 2.3). In this way, samples of 100 pmol synthetic Carmo-HrTH-I together with hemolymph for 0, 2-, 5-, 10- and 15-min incubation periods were generated. The following controls without synthetic Carmo-HrTH-I were included: (a) 30% acetonitrile with 0.1% TFA only and (b) hemolymph mixed with 30% acetonitrile with 0.1% TFA. Three biological repeats were carried out.

Additionally, Carmo-HrTH-II was added to Eppendorf tubes that contained inactivated hemolymph, that is, 10 µL hemolymph was mixed with 30 µL of 30% acetonitrile with 0.1% TFA to stop endogenous enzyme activity in the hemolymph, after which 100 pmol of Carmo-HrTH-II was added. The tubes were vortexed and left to stand at RT for 55 min, followed by a vortexing step, centrifugation and HPLC as described above. This was compared with

HPLC traces of a 0 min and a 3 min incubation of 100 pmol synthetic Carmo-HrTH-II into freshly drawn hemolymph from an adult stick insect.

2.6 | Synthetic peptides

Drs Runyu Mao and Ethan G. Goddard-Borger (The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia and the Department of Medical Biology, University of Melbourne) generously supplied the synthetic mannosylated peptide (Carmo-HrTH-I; Mao et al. [2021]) for our research. Synthetic nonmannosylated peptide (Carmo-HrTH-II) had previously been purchased from Peninsula Laboratories. A stock solution was prepared from synthetic Carmo-HrTH-I as follows: 0.2 mg of the peptide was reconstituted in 1 mL of 15% acetronitrile and an aqueous dilution (1:30) was made for further dilutions with distilled water. A similar preparation was followed with synthetic Carmo-HrTH-II.

3 | RESULTS

3.1 | Confirmation of the identity and biological activity of synthetic Carmo-HrTH-I

In a first series of experiments a CC extract from 2 gland pair equivalents of *C. morosus* was subjected to HPLC separation as outlined above and the fluorescence for Trp monitored. Substances that do not interact with the column material elute with the void volume at around 3–5 min, while two prominent fluorescence peaks elute later with the employed gradient (Figure 1a): the peak at 7 min represents the natural decapeptide material code-named Carmo-HrTH-I and the peak at 11 min is the natural decapeptide Carmo-HrTH-II in the stick insect CC. When 50 pmol of the chemically synthesized Carmo-HrTH-I (i.e., a Trp-mannosylated Carmo-HrTH-II) was run on the same HPLC column under the same conditions as the CC extract, the only fluorescent peak seen was at 7 min (Figure 1b). A mixture of 50 pmol of synthetic Carmo-HrTH-I and a CC extract from the equivalent of 2 glands from *C. morosus* resulted in a HPLC chromatogram that unequivocally showed coelution of the synthetic and natural Carmo-HrTH-I material (Figure 1c)—the resulting peak height at 7 min is double as high as in Figure 1a. This is one piece of evidence that the amino acid structure of the synthetic Carmo-HrTH-I matches that of the natural Carmo-HrTH-I produced in the CC.

Having established that the natural and synthetic Carmo-HrTH-I peptides have the same retention time, the next aim was to demonstrate that the synthetic peptide has biological activity. It was previously shown that the CC extract of *C. morosus* was very active in elevating lipids in the hemolymph of migratory locusts (Gäde, 1979, 1980). Thus, we injected into acceptor locusts, water (as negative control in which all test substances were dissolved), a locust CC extract (as positive control), the natural Carmo-HrTH peptides purified by RP-HPLC from a stick insect CC extract, as well as synthetic Carmo-HrTH peptides, and measured the total vanillin-positive material (=lipids) in the hemolymph. Whereas the control injection of water had no hyperlipemic effect, the locust CC extract, the stick insect natural HrTH peptides, as well as the synthetic hypertrehalosemic hormones elevated the hemolymph lipids significantly (Table 1).

A homologous set of bioassays was also performed with neck-ligated stick insects to test the biological activity of synthetic Carmo-HrTH-I. Due to not having access to a large colony of *C. morosus*, the acceptor insects were not very homogenous of age and physiological state but spanned from late 6th instar to about 10 days of adult life. This notwithstanding, the results showed clearly that water-injected control animals did not react significantly, whereas a low but statistically significant hypertrehalosemic effect was measured in response to injection of natural Carmo-HrTH-I, as well as with the synthetic Carmo-HrTH-I peptide (Table 2).

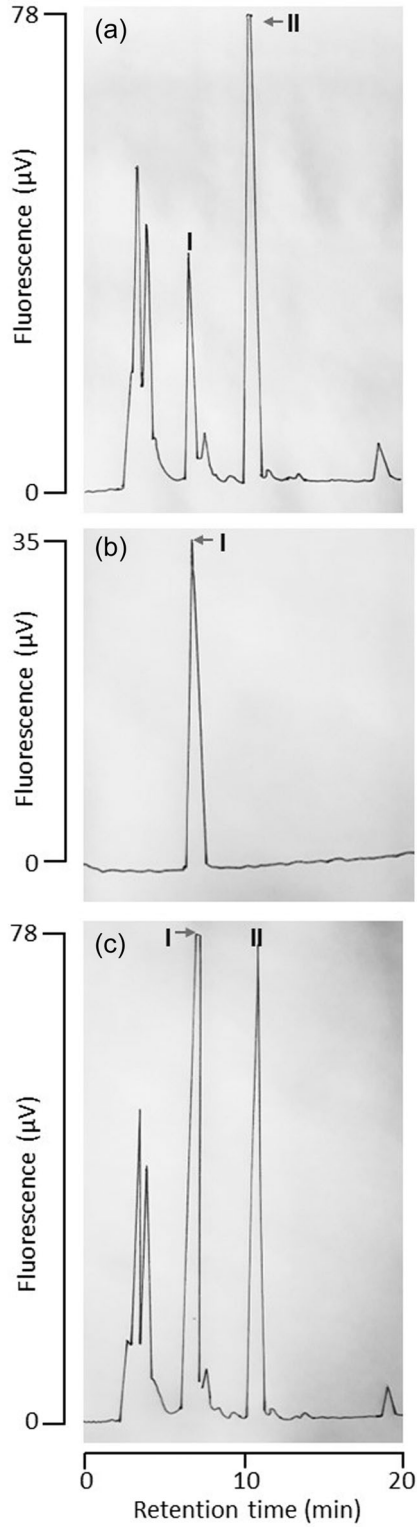

FIGURE 1 (See caption on next page)

3.2 | Biochemical parameters of Carmo-HrTH-I

3.2.1 | Heat stability of Carmo-HrTH-I

To examine pertinent physical and physiological parameters of the synthetic Trp-mannosylated peptide, which has identical chromatographic and biological properties as the naturally produced Carmo-HrTH-I peptide of *C. morosus*, further experiments tested the temperature sensitivity of the synthetic Carmo-HrTH-I peptide and its resistance to degradation in a natural biological milieu, such as hemolymph. When the synthetic HrTH peptides of *C. morosus* were exposed to 100°C for 30 min and then subjected to RP-HPLC, no difference in retention time, peak height and chromatographic profile was evident when compared with a peptide aliquot before heat treatment (Figure 2). These results attest to the heat stability of the HrTH synthetic peptides.

3.2.2 | Degradation of Carmo-HrTH-I in stick insect hemolymph in vitro

The next experiment was designed to supply information on the peptide breakdown in the hemolymph of *C. morosus* and whether an estimate of the half-life of the Carmo-HrTH-I molecule can be determined in the process. As depicted in Figure 3a, the peak height on RP-HPLC that corresponded to Carmo-HrTH-I continually

TABLE 1 Biological activity of a crude methanolic extract of corpora cardiaca (CC) from the migratory locust (*Locusta migratoria*), natural hypertrehalosemic hormones (HrTHs) extracted from the CC of the Indian stick insect (*Carausius morosus*) and the synthetic equivalents of the *C. morosus* HrTH peptides in an in vivo bioassay with *L. migratoria*.

Treatment	*n*	[Lipid]T_0min (μg/μL)	[Lipid]T_{90}min (μg/μL)	Difference (μg/μL)	*p*[a]
Distilled water	10	10.09 ± 2.02	9.74 ± 1.13	−0.36 ± 1.18	NS
Natural Carmo-HrTH-I (10 pmol)	9	9.32 ± 1.59	22.84 ± 5.06	13.47 ± 3.50	0.000003
Natural Carmo-HrTH-II (10 pmol)	6	9.35 ± 1.64	23.48 ± 3.04	14.12 ± 3.00	0.00004
Synthetic Carmo-HrTH-I (10 pmol)	12	10.07 ± 1.52	22.97 ± 5.34	12.90 ± 5.00	0.000001
Synthetic Carmo-HrTH-II (10 pmol)	6	10.05 ± 2.32	21.17 ± 5.29	11.12 ± 3.38	0.0002
L. migratoria CC (0.1 gland pair equivalent)	12	10.56 ± 2.30	24.30 ± 5.15	13.74 ± 4.48	0.0000002

Note: Data given as mean ± SD.

Abbreviations: NS, not significant; SD, standard deviation.

[a]A Paired *T*-test was applied to compare data before and after injection in the same individuals.

FIGURE 1 Chromatographic evidence of an identical profile for the natural C-mannosylated peptide hormone, Carmo-HrTH-I, from the Indian stick insect *Carausius morosus* and a chemically synthesized version of Carmo-HrTH-I. Reversed phase-HPLC separation of a crude methanolic extract of CC from 2 stick insects (a) revealed 2 major peaks identified as Carmo-HrTH-I (Peak I) and Carmo-HrTH-II (Peak II). (b) Synthetic Carmo-HrTH-I (50 pmol) and (c) coinjection of synthetic Carmo-HrTH-I (50 pmol) and 2 pCC from *C. morosus* under the same chromatographic conditions as in (a), reveal a common peak corresponding with the elution of natural Carmo-HrTH-I. The samples were applied to a Nucleosil C18 column, monitored by fluorescence (excitation 276 nm/emission 350 nm) and developed with a linear gradient of 0.11% TFA in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 43% to 53% B in 20 min at a flow rate of 1 mL/min. CC, corpora cardiaca; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

TABLE 2 Conspecific biological assays with ligated 6th instars and young adult Indian stick insect (*Carausius morosus*) to test the natural hypertrehalosemic hormone (Carmo-HrTH-I) extracted from the corpora cardiaca and the synthetic equivalent thereof.

Treatment	*n*	[Carbohydrate] T_0min (μg/μL)	[Carbohydrate] T_{90}min (μg/μL)	Difference (μg/μL)	*p**
Distilled water	10	6.78 ± 1.16	6.87 ± 1.48	0.09 ± 0.68	NS
Natural Carmo-HrTH-I (10 pmol)	6	6.46 ± 1.65	8.06 ± 1.62	1.60 ± 1.28	0.01
Synthetic Carmo-HrTH-I (10 pmol)	10	7.38 ± 1.60	10.40 ± 2.01	3.02 ± 1.00	0.00003

Note: Data given as mean ± SD.

Abbreviations: NS, not significant; SD, standard deviation.

*A Paired *T*-test was applied to compare data before and after injection in the same individuals.

decreased with increasing incubation time of the peptide in the hemolymph. Almost nothing of the 100 pmol Carmo-HrTH-I is left after 15 min in hemolymph, and the half-life of the peptide can be determined around 5 min. The respective HPLC traces show that the controls had no fluorescent peaks (Figure 3b: solvents without peptide) or only a peak corresponding to the void volume (Figure 3c: a mixture of hemolymph and solvents). On the other hand, the chromatogram of the 0 min incubated sample, that is, 100 pmol peptide mixed with hemolymph at the start of the experiment, revealed a high fluorescent peak at 7 min (Figure 3d) and this peak corresponding to the Carmo-HrTH-I peptide was almost negligible after 15 min incubation in hemolymph (Figure 3e). We deduced that this loss in the amount of Carmo-HrTH-I over time was due specifically to enzymatic activity in the complex hemolymph milieu and not merely due to peptide adhesion to the wall of the tube or to adhesion to the injection syringe. This is borne out in part by the results in Section 3.2.1 where the HrTH peptides did not show a reduction in peak height (proxy for peptide quantity) after a period of 30 min in a tube in the absence of hemolymph. In addition, peptide adhesion was controlled for in experiments with the more-hydrophobic Carmo-HrTH-II (Figure 4): no sizeable difference in peak height was obtained from a mixture of hemolymph and acetonitrile-TFA to which Carmo-HrTH-II was then added (i.e., peptide in enzyme-inactivated hemolymph) and left to incubate at RT for 55 min (Figure 4a), versus a mixture of hemolymph and Carmo-HrTH-II to which acetonitrile-TFA was then added, thus a 0 min peptide incubation in hemolymph (Figure 4b), whereas a 55% reduction in peak height was evident after Carmo-HrTH-II was incubated with hemolymph for 3 min before enzyme inactivation by the addition of acetonitrile-TFA (Figure 4c).

In the experiments with hemolymph exposure of Carmo-HrTH-I, in none of the resulting chromatograms (see Figure 3d,e) can we detect a fluorescent peak coincident with the retention time of Carmo-HrTH-II (11 min), suggesting that the presumed enzymatic degradation of Carmo-HrTH-I does not entail the mere removal of the glycolytic bond at Trp to convert Carmo-HrTH-I into Carmo-HrTH-II (the non-glycosylated peptide). It is further deduced that the glycolytic bond at Trp is stable, and that Carmo-HrTH-I was enzymatically degraded into more-polar fragments not detectable in the HPLC set-up.

3.2.3 | HrTH release from CC by depolarization

To confirm that Carmo-HrTH-I is indeed a true hormone that is released from the CC under conditions of depolarization, in vitro experiments were performed with *C. morosus* CCs that were carefully removed from the insect body to remain intact; the saline solutions in which the CC were successively incubated in were retained for RP-HPLC (see Materials and Methods). The saline itself shows no fluorescent material before incubation with CC

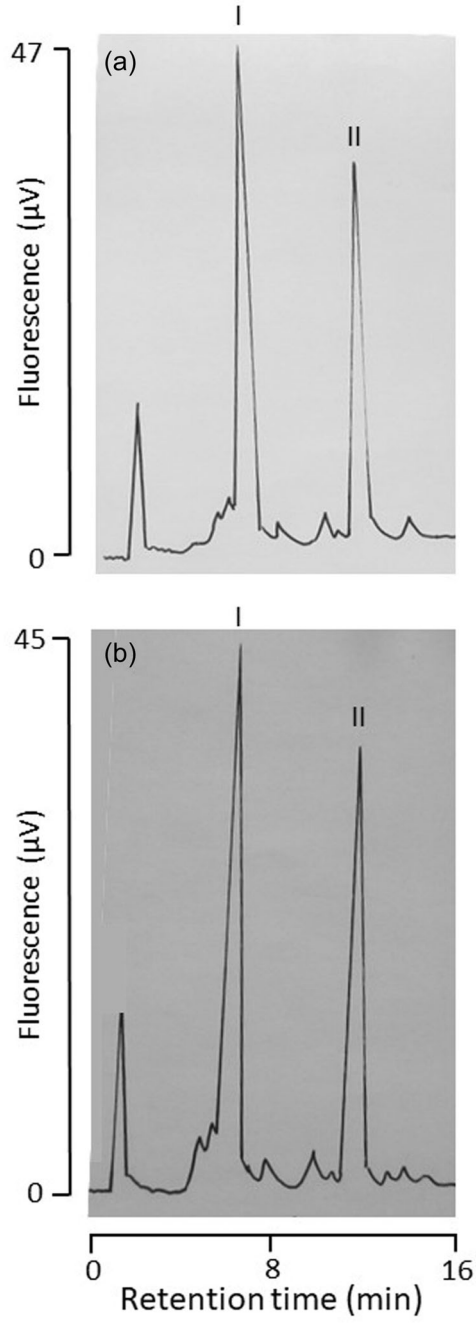

FIGURE 2 Chromatographic evidence of the stability of the synthetic C-mannosylated peptide hormone, Carmo-HrTH-I, and the synthetic non-mannosylated peptide hormone, Carmo-HrTH-II, to heat. The RP-HPLC profile of 50 pmol untreated chemically synthesized peptides (a) and following an incubation at 100°C for 30 min (b). For RP-HPLC, the synthetic peptides (heat-treated and untreated) were applied to a Nucleosil C18 column, monitored by fluorescence (excitation 276 nm/emission 350 nm) and developed with a linear gradient of 0.11% TFA in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 43% to 53% B in 20 min at a flow rate of 1 mL/min. RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

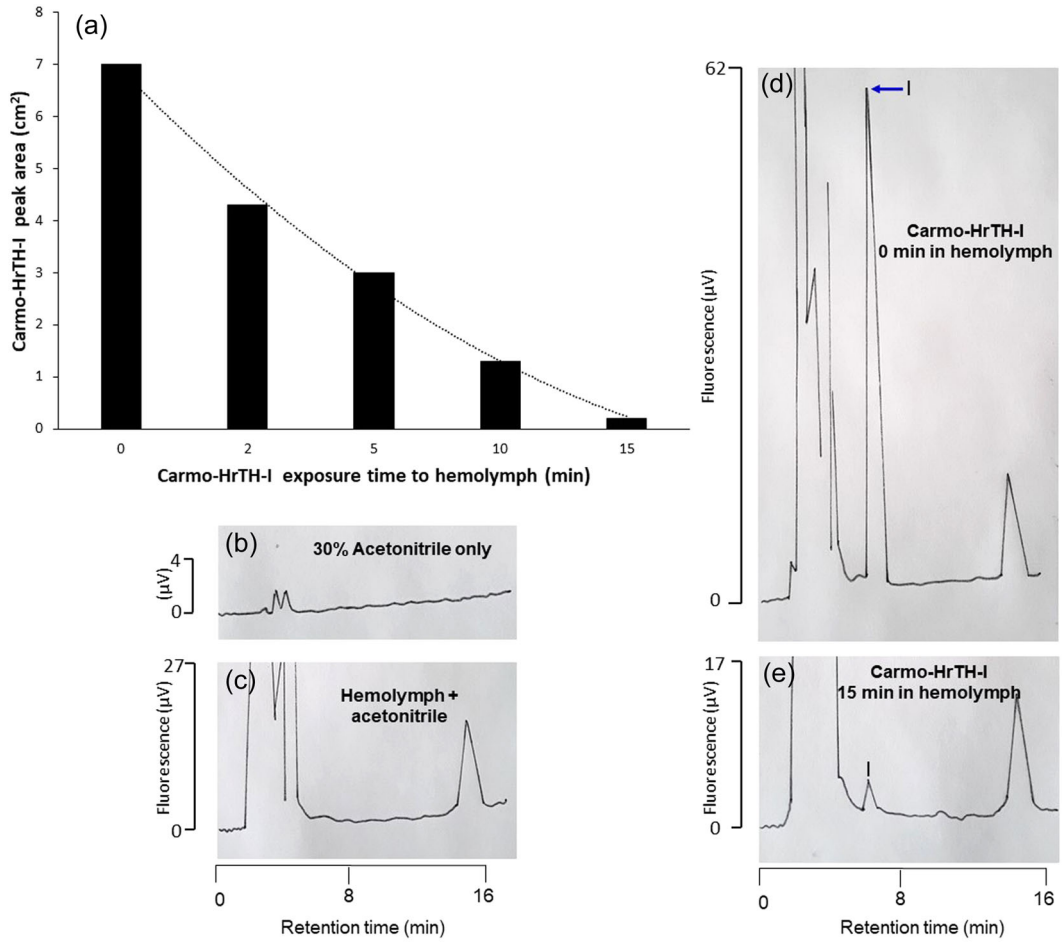

FIGURE 3 The breakdown of synthetic Carmo-HrTH-I in vitro in hemolymph from adult Indian stick insects, *Carausius morosus*. Freshly drawn hemolymph was added to 100 pmol of the synthetic peptides and left to incubate at RT for exactly timed periods (range 0–15 min). A "stop" solution of acetonitrile and TFA was added at the end of the incubation period. The samples were centrifuged and then applied to RP-HPLC, monitoring by fluorescence (excitation 276 nm/emission 350 nm). The resulting Carmo-HrTH peak areas were calculated as a measure of peptide quantity remaining after incubation (a). (b) And (c) represent RP-HPLC chromatograms of control solutions, that is, the "stop" solution (b) and stick insect hemolymph (c), both devoid of Carmo-HrTH peaks. (d) And (e) show the synthetic Carmo-HrTH-I (Peak I) on RP-HPLC chromatograms following 0 and 15 min incubation in hemolymph, respectively. For RP-HPLC, the samples were applied to a Nucleosil C18 column, monitored by fluorescence (excitation 276 nm/emission 350 nm) and developed with a linear gradient of 0.11% TFA in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 43% to 53% B in 20 min at a flow rate of 1 mL/min. RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

(Figure 5a). Following incubation of the CC in saline with a low potassium concentration, the collected saline showed very small peaks at 7.0 and 11.0 min on fluorescence trace (Figure 5b), representing constitutively released Carmo-HrTH-I and II, respectively. Elevation of the potassium concentration in the saline to membrane depolarization levels resulted in a fluorescent trace that clearly showed higher peaks for Carmo-HrTH-I and -II in the collected saline (Figure 5c).

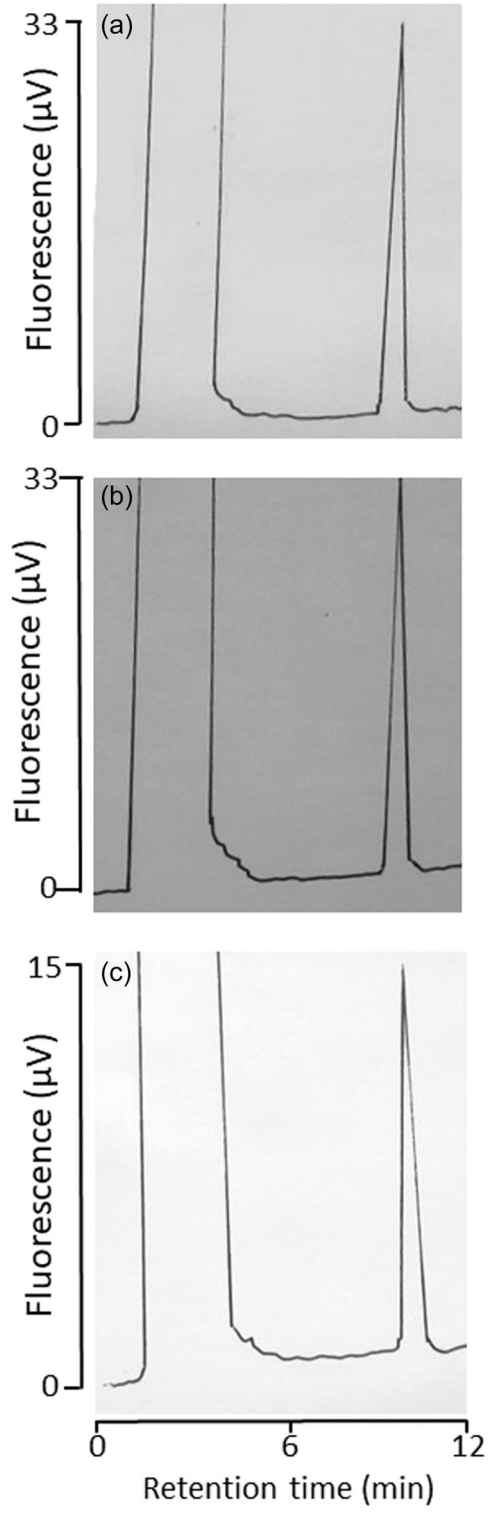

FIGURE 4 (See caption on next page)

4 | DISCUSSION

Imperative for the performance of the current study was the work by Mao et al. (2021) who found not only a convenient and reliable method to synthesize the unusually modified amino acid tryptophan with a mannose sugar moiety but could also develop a method how to incorporate such a modified C-mannosylated Trp residue into a peptide using automated solid phase peptide synthesis. With such a method available, milligram amounts of a decapeptide with a C-mannosylated Trp residue, which is naturally produced in the CC cells of the Indian stick insect *C. morosus* in small quantities (Gäde et al., 1992; Munte et al., 2008), could be synthesized (Mao et al., 2021). The availability of sufficient synthetic Carmo-HrTH-I made it possible for us to answer a number of important questions that had arisen since the discovery of two decapeptide members of the AKH peptide family in the stick insect: an unmodified and an otherwise identical C-mannosylated peptide; both peptides displayed adipokinetic activity in migratory locusts and a hypertrehalosemic effect in American cockroaches (thus both heterologous assays), as well as causing hypertrehalosemia in the stick insect itself, that is, in a conspecific bioassay (Gäde & Lohr, 1982; Gäde, 1979).

After we had established in the current study that the newly synthesized peptide Carmo-HrTH-I was identical to the natural peptide in retention time (see Figure 1) and was also stable to heat inactivation, we had to show that the peptide was active in a few bioassays. Clearly, a low dose of Carmo-HrTH-I was active in mobilizing lipids in locusts, as well as inducing hypertrehalosemia in ligated stick insects (Tables 1 and 2). There was, however, still a "snag" in the whole experimental set up, that could put a spanner in the work. What if the bond between the mannose and Trp is broken enzymatically in the hemolymph to convert Carmo-HrTH-I to Carmo-HrTH-II? Since we inject the synthetic Carmo-HrTH-I we assume that it is stable in the hemolymph environment and that it binds to an AKH receptor in the insect, but this was never shown directly or indirectly. If Carmo-HrTH-I was, however, not stable at this bond, the resulting peptide would be Carmo-HrTH-II which we had shown previously to have biological activity (Katali et al., 2020). Hence, we designed an in vitro experiment using Carmo-HrTH-I and hemolymph and show that, even if breakdown occurs, no Carmo-HrTH-II is formed. The incubation of Carmo-HrTH-I with hemolymph from *C. morosus* showed two unequivocal results:

1. Carmo-HrTH-II (retention time of 11 min) was not detected by RP-HPLC during the incubation time of up to 15 min.
2. Carmo-HrTH-I (retention time of 7 min) was enzymatically broken down and hardly detectable by RP-HPLC after 15 min incubation. Calculations point to a half-life of this peptide of about 5 min (see Figure 3). This is somewhat longer half-life than published for the endogenous octapeptide Grybi-AKH (2 min) in the cricket *Gryllus bimaculatus* (Woodring et al., 2002). In resting migratory locusts, the half-life for each of the three endogenous AKHs are given as 51, 40 and 5 min, respectively for Locmi-AKH-I, -II and -III (Oudejans et al., 1996), thus much longer than Carmo-HrTH-I.

FIGURE 4 The failure of inactivated hemolymph from adult Indian stick insects, *Carausius morosus*, to degrade synthetic Carmo-HrTH-II in vitro. Freshly drawn hemolymph was added to a "stop" solution of acetonitrile and TFA to inactivate peptidases; 100 pmol of the synthetic peptide was added to the inactivated hemolymph and left to incubate at RT for 55 min before centrifugation and RP-HPLC (a). Two further samples of 100 pmol of the synthetic peptide were incubated at RT in freshly drawn hemolymph for 0 min (b) and 3 min (c) before adding the "stop" solution. The samples were centrifuged and applied to RP-HPLC (Nucleosil C18 column), monitoring by fluorescence (excitation 276 nm/emission 350 nm). A linear gradient of 0.11% TFA in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 43% to 53% B in 20 min at a flow rate of 1 mL/min was developed. The resulting Carmo-HrTH-II peak area and height were assessed as a measure of peptide quantity remaining after incubation with active or inactivated hemolymph. RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

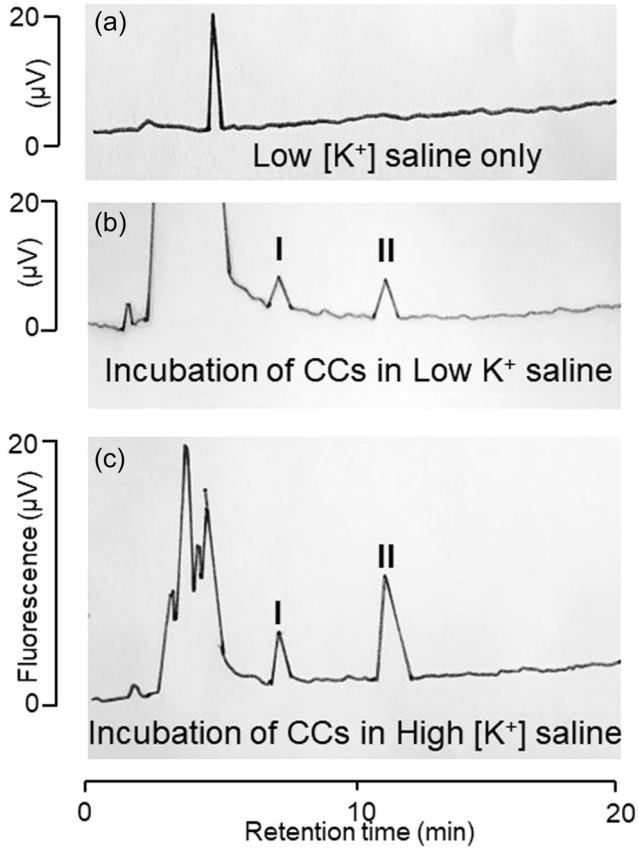

FIGURE 5 Chromatographic evidence for the release of the natural C-mannosylated peptide hormone, Carmo-HrTH-I, and the natural non-mannosylated peptide hormone, Carmo-HrTH-II, from corpora cardiaca of the Indian stick insect *Carausius morosus* in vitro upon incubation with high potassium saline. RP-HPLC chromatograms are shown of (a) Control run: injection of low potassium saline onto the column; (b) injection of low potassium saline in which CCs from 10 adult stick insects had been incubated for 30 min at 25°C; (c) injection of high potassium saline in which CCs from 10 adult stick insects had been incubated for 30 min at 25°C. Carmo-HrTH-I and -II peaks are labeled I and II, respectively. For RP-HPLC, the saline samples were applied to a Nucleosil C18 column, monitored by fluorescence (excitation 276 nm/emission 350 nm) and developed with a linear gradient of 0.11% TFA in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 43% to 53% B in 20 min at a flow rate of 1 mL/min. CC, corpora cardiaca; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

Thus, the C-glycosidic bond of Carmo-HrTH-I seems to be strong and not affected by enzymatic breakdown by any enzyme residing in the hemolymph of *C. morosus*; the exact target for an enzyme at the remaining peptide bonds is not clear yet and needs further experimentation. Peptide degradation during incubation of Carmo-HrTH-II in stick insect hemolymph was also demonstrated in the current study. As was the case with Carmo-HrTH-I, the breakdown products were not detectable with our HPLC set-up, which targets the Trp residue. We assume that the peptides are cleaved into more-polar fragments for which a different solvent gradient with UV detection may be required for peak detection.

Further evidence that Carmo-HrTH-I is, indeed, a neurohormone that is released into circulation from the retrocerebral neuroendocrine site in the stick insect comes from classic in vitro experiments in this study where the release of Carmo-HrTH-I from neurosecretory cells of the CC was induced through depolarization of the cell

membrane due to high K^+ in the incubating medium. Carmo-HrTH-I is, thus, shown to be releasable and is not an artifact.

Currently, we favor the following working hypothesis regarding the hypertrehalosemic hormones in the Indian stick insect: the HrTH-producing neurons in the corpus cardiacum contain mRNA that is translated into one copy of the HrTH precursor which will give rise to two mature decapeptides. This biosynthesis hypothesis is supported by transcriptomic and neuropeptidomic data where only one "AKH" neuropeptide precursor was sequenced from *C. morosus*, whereas two mature "AKH" neuropeptides were identified by mass spectrometry as Carmo-HrTH-II and Carmo-HrTH-I (Liessem et al., 2018). It is long known that proteolytic processing of the prohormones to bioactive (mature) AKHs takes place in a post-transGolgi compartment in the CC cells, presumably in the secretory granules (see Diederen et al., 2002); we assume, therefore, that this may also be the site where Carmo-HrTH-I is formed via an additional post-translation modification step of Carmo-HrTH-II to result in a C-mannosylated Trp at position 8. We further postulate that upon the correct stimulus when CC cells are depolarized, both mature peptides are released into the hemolymph and travel to the single AKH receptor known from the stick insect (Birgul Iyison et al., 2020), for example, in the fat body. In insect species that do not practice thanatosis (i.e., do not feign death as an antipredator strategy), the AKH or HrTH peptide will bind and activate the GPCR, leading to a signal transduction cascade, activation of glycogen phosphorylase and finally, after some more enzymatic steps, the release of trehalose from the fat body into the hemolymph can be monitored as an increase in carbohydrate concentration (see Gäde & Auerswald, 2003). In parallel to this, AKH/HrTH peptides also bind to receptors on the insect heart resulting in an increase of the heartbeat frequency and faster pumping of hemolymph through the body as evidenced by previous experiments (see Katali et al., 2020; Marco et al., 2018).

In the case of stick insects, including *C. morosus*, however (and possibly in other insects with thanatosis display), the injection of endogenous AKH/HrTH peptides do not lead to the expected end result of hypertrehalosemia (even though glycogen phosphorylase is activated) unless a ligature is applied to the stick insect thereby limiting circulation between the head and the rest of the body (Gäde & Lohr, 1982; Gäde, 1989). Similarly, the usual cardioaccelerator effect is only present in ligated *C. morosus* (Marco et al., 2018) and in decapitated Vietnamese stick insects, *Baculum extradentatum* (Malik et al., 2012). It would seem, therefore, that the AKH signaling system in the stress response of fight or flight may be naturally "disrupted" in the stick insect to enable its survival strategy of remaining motionless in the presence of potential predators.

AUTHOR CONTRIBUTION

Gerd Gäde: Conceptualization (lead), experimentation (HPLC, bioassay), data curation (equal), validation (equal), writing—original draft (equal), funding acquisition, resources, project administration. **Heather G. Marco**: Conceptualization (supportive), experimentation (bioassays, stability, and release), data curation (equal), validation (equal), writing—original draft (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the main article.

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