ShK toxin from the sea anemone *Stichodactyla helianthus* is a 35-residue protein that binds to the Kv1.3 ion channel with high affinity. Recently we determined the X-ray structure of ShK toxin by racemic crystallography, in the course of which we discovered that D-ShK has a near-background IC₅₀ value ~50,000 times lower than that of the L-ShK toxin. This lack of activity was at odds with previously reported results for an ShK diastereomer designated D-allo-ShK, for which significant biological activity had been observed in a similar receptor-blocking assay. As reported, D-allo-ShK was made up of D-amino acids, but with retention of the natural stereochemistry of the chiral side chains of the Ile and Thr residues, *i.e.* containing D-allo-Ile and D-allo-Thr along with D-amino acids and glycine. To understand its apparent biological activity, we set out to chemically synthesize D-allo-ShK and determine its X-ray structure byracemic crystallography. Using validated allo-Thr and allo-Ile, both L-allo-ShK and D-allo-ShK polypeptide chains were prepared by total chemical synthesis. Neither the L-allo-ShK nor the D-allo-ShK polypeptides folded, whereas both L-ShK and D-ShK folded smoothly under the same conditions. Re-examination of NMR spectra of the previously reported D-allo-ShK protein revealed that diagnostic Thr and Ile signals were the same as for authentic D-ShK. On the basis of these results, we conclude that the previously reported D-allo-ShK was in fact L-ShK, the true enantiomer of natural L-ShK toxin, and that the apparent biological activity may have arisen from inadvertent contamination with trace amounts of L-ShK toxin.

It has been shown numerous times that, as would be expected from symmetry considerations, a polypeptide chain made up of D-amino acids (and achiral Gly) that has the same amino acid sequence as the natural L-protein will fold to form a D-protein molecule that is the mirror image of the corresponding L-protein (6, 7). Recently we reported the convergent total synthesis of wild-type ShK toxin and its mirror image form D-ShK, for determination of the ShK structure by racemic protein crystallography (8). During that work we demonstrated that our synthetic L-ShK, of the same L-protein chirality as ShK toxin found in nature, is biologically active in functional blockade of hKv1.3 currents using the cut-open oocyte voltage clamp method (9). Synthetic L-ShK blocked potassium currents in a concentration-dependent manner, with an IC₅₀ value of ~250 pM. This result was consistent with other reports using the same assay method (10).

In the same paper, we reported that synthetic D-ShK toxin was biologically inactive: D-ShK protein, the unnatural mirror image protein, had a near-background IC₅₀ value ~50,000 times lower than the L-ShK toxin (8). This observed lack of affinity of D-ShK for the target Kv1.3 ion channel is as expected, because chirality is believed to be of great importance in protein-protein interactions; a mirror image protein (*i.e.* a D-protein) molecule would not be expected to bind to the natural protein target of the corresponding L-protein (11, 12). Surprisingly, it had been reported previously that a diastereomer of the mirror image D-ShK protein, designated d-allo-ShK, retained some biological activity and bound to the Kv1.3 ion channel (13). This d-allo-ShK protein was described as being made up of D-amino acids, but with retention of the natural stereochemistry of the chiral side chains of the Ile and Thr residues, *i.e.* made up of d-allo-Ile and d-allo-Thr residues, along with D-amino acids and glycine (13). Isoleucine and threonine are the only two genetically encoded amino acids bearing two chiral centers: one at the α carbon atom and another at the β carbon atom. Inversion of the stereochemistry only at the α carbon does not convert L-Ile and L-Thr to D-Ile and D-Thr. Rather, the diastereomeric amino acids d-allo-Ile and d-allo-Thr are generated. Similarly, inversion of the α carbon stereochemistry in D-Ile and D-Thr generates L-allo-Ile and L-allo-Thr (13) (Scheme 1).

Beeton *et al.* (13) reported that a d-allo-ShK polypeptide had folding properties similar to those of the ShK polypeptide chain. The authors performed the functional blocking assay of Kv1.3 expressed in L929 cells in the whole cell configuration of patch clamp technique. They reported that d-allo-ShK blocked
Kv1.3 with a $K_a$ value of 36 nm, whereas wild-type ShK blocked Kv1.3 with $K_a$ 13 pm in the same assay. That is, $\delta$-allo-ShK apparently had ~2800-fold lower affinity for Kv1.3 than wild-type ShK protein. The authors also reported that both $\delta$-allo-ShK and wild-type ShK were 2-fold selective for Kv1.3 channel over Kv1.1 channel (13).

The apparent lack of activity of our $\delta$-ShK was at odds with these reported results for $\delta$-allo-ShK. We have therefore chemically synthesized authentic $\delta$-allo-ShK polypeptide chain and reinvestigated its folding properties and biological activity. We also intended to use racemic crystallography to determine the X-ray structure of the $\delta$-allo-ShK protein molecule, in order to correlate the folded structure of this unusual analog with its reported activity. For that reason, we set out to make both $\delta$-allo-ShK and its mirror image form, $\lambda$-allo-ShK.

### Results

#### Authentication of $\lambda$-Ile and $\lambda$-Thr

We previously reported the development of methods for the rigorous characterization of the amino acids $\delta$-allo-Ile, $\lambda$-allo-Ile, and $\delta$-allo-Thr, and $\lambda$-allo-Thr (14). Analytical data, including optical rotations, NMR measurements, X-ray structures, and chromatographic separations of all four Ile and all four Thr amino acids as peptide diastereomers, confirmed the identities of the Ile and the Thr enantiomers and diastereomers that were used in the syntheses of the various ShK analog polypeptide chains in the work reported here.

#### Total chemical synthesis of $\delta$-allo-ShK polypeptide chains

The amino acid sequence of ShK is shown in Scheme 2. The synthetic strategy for the preparation of the $\delta$-allo-ShK polypeptide chain was the same as that used for the total synthesis of ShK toxin as previously described (8). Unprotected peptide segments 1-Gln$^{16}$,thioester and Cys$^{17}$-35 were prepared by in situ neutralization t-butoxycarbonyl (Boc)$^2$ chemistry solid phase peptide synthesis (15). After purification by preparative HPLC and characterization by LC-MS, the two peptide segments were covalently condensed by native chemical ligation (15). Instead, each analog formed a complex mixture of components eluting later than the previously characterized correctly folded ShK. In each case, scrutiny of the LC-MS data using expected m/z values revealed only minute traces of a product corresponding to the calculated mass of a correctly folded allo-ShK protein product.

Surprisingly, none of the diastereomer allo-ShK polypeptide chains gave a discrete correctly folded protein product, even after 40 h (Fig. 3, b–e). Instead, each analog formed a complex mixture of components eluting later than the previously characterized correctly folded ShK. In each case, scrutiny of the LC-MS data using expected m/z values revealed only minute traces of a product corresponding to the calculated mass of a correctly folded allo-ShK protein product.

After we found that air oxidation was not able to produce defined folded products for any of the allo-ShK polypeptide
chains, we investigated the use of a conventional redox pair consisting of cysteine and cystine/H$_2$HCl to fold these polypeptides at pH 8.0. However, in our hands, the redox pair folding conditions also did not give a defined folded product for any of the allo-ShK polypeptide chains.

In related work, using L-allo-Ile and L-allo-Thr amino acids authenticated by the same methods used here, we found that in contrast to the inability to fold the all-allo-amino acid ShK polypeptide chains, inversion of the side chain stereochemistry of individual Thr or Ile residues in ShK gave polypeptide chains that folded to give protein products with the same three-dimensional structure as wild-type ShK (14). Introduction of single allo-amino acids had only minimal effect on the affinity of the resulting protein diastereomers for the Kv1.3 channel (14).

**Discussion**

The previously reported D-allo-ShK was actually D-ShK

In their 2008 paper (13), Beeton et al. reported that a D-allo-ShK polypeptide chain could be folded and that the resulting D-allo-ShK protein molecule retained significant biological activity. In the work reported here, we found that allo-ShK polypeptide chains did not fold to give a protein molecule of defined three-dimensional structure under the conditions used by Beeton et al. As we had reported previously, the D-ShK-CONH$_2$ polypeptide chain folded readily, equally as well as the L-ShK polypeptide chain had in our previous studies (8), to give a single folded D-protein molecule. Based on these studies of authentic D-allo-ShK polypeptide chains, we suspected that the D-allo-ShK reported by Beeton et al. was in fact simply the mirror image D-ShK protein, because the reported folding properties of their D-allo-ShK polypeptide are the same as we observed for wild-type ShK and for D-ShK-CONH$_2$ polypeptide chains.

To further investigate this possibility, we obtained NMR spectra of our authentic synthetic D-ShK (8) and reexamined the NMR spectrum previously obtained for the D-allo-ShK (13). The comparison of these two spectra showed that D-ShK and the reported D-allo-ShK were essentially identical. To be specific, the H$_{a}$ chemical shifts of D-Ile$^{4}$ and D-Ile$^{7}$ in our D-ShK are 4.67 and 4.77 ppm (Fig. 4 and Table 1). The H$_{a}$ chemical shifts of D-Ile$^{4}$ and D-Ile$^{7}$ in the reported D-allo-ShK are 4.66 and 4.76 (13), which are identical (within 0.01 ppm) to the corresponding chemical shifts in the authentic D-ShK and L-ShK (17). If these Ile residues had been D-allo, their H$_{a}$ chemical shifts would have differed from those in L-ShK.$^{3}$ Considering

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$^{3}$ David Fox, personal communication.
the inability to fold allo-ShK polypeptide chains in conjunction with these NMR data, we conclude that the D-allo-ShK reported by Beeton et al. was actually the mirror image D-ShK protein.

Where did the reported biological activity of D-allo-ShK come from?

As for the reported biological activity of the putative D-allo-ShK, we suspect that it may have come from contamination by traces of wild-type ShK toxin. Wild-type ShK is highly potent against its target Kv1.3 with an IC_{50} in the picomolar range, and wild-type ShK is 2-fold selective for Kv1.3 over Kv1.1(10). The reported D-allo-ShK was also 2-fold selective for Kv1.3 over Kv1.1(13). This selectivity supports the possibility of inadvertent contamination with wild-type ShK, which even in trace amounts could give the levels of activity reported for the putative D-allo-ShK.
We have seen a similar contamination effect in our own hands (8). In that work, when we first tested our synthetic D-ShK for activity, we observed it to block Kv1.3 with an IC50 of ~450 nm, which was ~2000-fold less active than wild-type ShK in the same assay. This ~2000-fold activity difference is comparable with ~2800-fold lower affinity for Kv1.3 than wild-type ShK reported by Beeton et al. (13) for D-allo-ShK. We suspected that the apparent activity of our synthetic D-ShK (8) may have come from contamination by trace amounts of wild-type ShK, because we had purified synthetic D-ShK using the same reverse phase HPLC column on which wild-type synthetic ShK had been purified.

Although the preparative HPLC column had been washed intensively between purification of the different compounds, there is no guarantee that trace cross-contamination cannot occur. Unfortunately, even the most stringent wash procedures of HPLC columns do not completely remove all traces of samples run previously. It has been reported that subsequent purification of a different peptide or protein can displace traces of previously purified materials, a phenomenon known as “sweep up” (18, 19). Even trace contamination with a peptide or protein having picomolar affinity can lead to spurious activity results when the same preparative column is used repeatedly, as is often the case in most research laboratories.

For that reason, in our previous work (8) we resynthesized the D-ShK protein from scratch. The synthetic peptide segments, the ligated full-length polypeptide, and the folded D-ShK protein were purified using fresh columns that had never been exposed to wild-type ShK or to synthetic L-ShK. We then performed functional assays on the newly prepared D-ShK and found that synthetic D-ShK had essentially only background levels of Kv1.3 blocking activity (~1/50,000 that of wild-type ShK) (8). These data suggested that cross-contamination by trace amounts of wild-type ShK toxin can occur readily, and it is likely that such inadvertent cross-contamination may have occurred for the previously reported D-allo-ShK (13).

Concluding statement

In summary, we have used total chemical synthesis to prepare authentic D-allo-ShK, D-allo-ShK-CONH2, L-allo-ShK, and L-allo-ShK-CONH2 polypeptide chains in which the side chain stereochemical configurations of all Thr and Ile residues were inverted. In our hands, none of these allo-amino acid-containing polypeptide chains could be folded into a protein molecule of defined tertiary structure, whereas corresponding control ShK polypeptide chains without allo-amino acids folded readily.

These results cast doubt on the previously reported biological activity of D-allo-ShK. Based on the assay data we observed with our first—contaminated—batch of D-ShK (8), and on the lack-of-folding observed for D-allo-ShK polypeptide chains in this study, together with re-examination of protein NMR data, we conclude that the previously reported D-allo-ShK was in fact D-ShK, the mirror image form of ShK. We suggest that the apparent biological activity of that putative D-allo-ShK protein

### Table 1

| Residues | H  | CαH | CβH | Others |
|----------|----|-----|-----|--------|
|          | ppm| ppm | ppm | ppm    |
| Arg1     | 8.53| 4.17| 2.34| 2.89   | H8 7.09 | H6 6.85 |
| Ser2     | 8.32| 3.87| 1.45|        | H8 7.33 | H6 6.80 |
| Cys3     | 8.04| 4.01| 3.34| 2.60   | H8 7.50 | H6 6.94 |
| Ile4     | 8.11| 3.93| 2.25| 1.75   | H8 7.53 | H6 3.34 |
| Thr5     | 8.25| 4.46| 1.79| 1.49   | H8 7.10 | H6 3.24 |
| Lys6     | 7.52| 4.75| 3.56| 3.44   | H8 7.37 |
| Cys7     | 7.51| 5.33| 3.21| 2.56   | H8 7.13 | H6 2.20 |
| Arg8     | 8.45| 3.93| 1.84| 1.63   | H8 7.14 |
| Leu9     | 7.23| 4.17| 1.84|        | H8 7.32 |
| Ser10    | 8.07| 4.48| 2.03| 1.86   | H8 7.46 |
| Ser11    | 8.14| 4.43| 2.29| 1.94   | H8 7.17 |
| Arg12    | 7.99| 5.05| 3.27| 2.91   | H8 7.35 |
| Thr13    | 7.24| 4.38|    |        |        |
| Ala14    | 8.88| 3.99|    |        |        |
| Phe15    | 8.53| 4.17| 3.24| 2.89   | H8 7.09 | H6 6.85 |
| Glu16    | 7.83| 4.21| 1.99| 1.55   | H8 2.35 |
| Cys17    | 8.52| 4.24| 3.20| 2.93   | H8 6.56 |
| Lys18    | 7.55| 4.03| 1.57| 1.41   | H8 6.40 |
| His19    | 7.79| 4.48| 3.09| 2.35   | H8 6.56 |
| Ser20    | 8.37| 5.02| 4.08| 3.87   | H8 6.56 |
| Met21    | 9.15| 4.10| 2.66| 2.13   | H8 2.57 |
| Lys22    | 8.32| 3.87| 1.45|        | H8 1.69 |
| Tyr23    | 8.04| 4.01| 3.34| 2.60   | H8 7.50 |
| Arg24    | 8.11| 3.93| 2.25| 1.75   | H8 1.53 |
| Leu25    | 8.25| 4.46| 1.79| 1.49   | H8 1.70 |
| Ser26    | 7.52| 4.75| 3.56| 3.44   | H8 0.88 |
| Phe27    | 7.51| 5.33| 3.21| 2.56   | H8 6.20 |
| Cys28    | 8.70| 5.84| 3.31| 3.16   | H8 7.19 |
| Arg29    | 8.45| 3.93| 1.84| 1.63   | H8 1.49 |
| Lys30    | 7.23| 4.17| 1.84|        | H8 1.49 |
| Thr31    | 10.87| 3.87| 4.07|        | H8 1.31 |
| Cys32    | 9.15| 4.79| 3.34| 2.92   | H8 1.32 |
| Gly33    | 7.87| 4.08|    |        |        |
| Thr34    | 8.75| 4.17| 4.43|        | H8 1.15 |
| Cys35    | 7.79| 4.33| 2.95| 3.35   |        |
Biological activity of d-allo-ShK toxin

(13) arose from inadvertent contamination with native ShK toxin during purification.

Experimental procedures

Reagents

2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and N\textsuperscript{α}-Boc-protected L-amino acids (Peptide Institute, Osaka, Japan) were obtained from Peptides International (Louisville, KY). N\textsuperscript{α}-Boc protected D-amino acids were obtained from the Peptide Institute (Osaka, Japan). The side-chain protecting groups used were Arg(Tos), Asp(OcHex), Asn(Xan), Cys(4-CH\textsubscript{3}Bzl), His(DNP), Lys(2CI-Z), Ser(Bzl), Thr(Bzl), and Tyr(2Br-Z). Boc-L-allo-Ile-OH was obtained from Peptides International. Boc-L-allo-Thr(OMe)-OH, Boc-L-allo-Thr(Bzl)-OH, and Boc-L-allo-Thr(Bzl)-OH were prepared in house as previously described (14). Aminomethyl-resin (1.07 mmol/gram) was prepared from Biobeads S-X1 (Bio-Rad) (20). Boc-L-Cys-OCH\textsubscript{2}-phenylacetic acid and Boc-L-Ala-OCH\textsubscript{2}-phenylacetic acid were purchased from NeoMPS (Strasbourg, France). Boc-D-Cys-OCH\textsubscript{2}-phenylacetic acid was prepared in house according to published procedures (20). N,N-Diisopropylethylamine was obtained from Applied Biosystems. Diethyl ether, dichloromethane, N,N-dimethylformamide, HPLC-grade acetonitrile, and guanidine hydrochloride were purchased from Fisher. TFA was obtained from Halocarbon Products. HF was purchased from Matheson. All other reagents were purchased from Sigma-Aldrich and were of the purest grade available.

Peptide synthesis

Peptide segments were synthesized on a 0.2-mmol scale using manual in situ neutralization Boc chemistry protocols for stepwise solid phase peptide synthesis (15). Peptide-thioesters were synthesized on trityl-SCH\textsubscript{2}CH\textsubscript{2}CO-Ala-OCH\textsubscript{2}-Pam-resin (21). Peptides with a free α-COOH were synthesized on Boc-Cys-OCH\textsubscript{2}-Pam-resin prepared from aminomethyl-resin. Peptide segments with a ω-CO-NH\textsubscript{2} were first synthesized as peptide thioesters and then reacted with ammonium acetate at pH 9.0 for C-terminal amidation. After assembly of the target peptide and removal of the N-terminal N\textsuperscript{α}-Boc group, each peptide was cleaved from the resin and simultaneously protected by treatment at 0 °C for 1 h with anhydrous HF containing 5% (v/v) p-cresol as scavenger. After removal of HF by evaporation under reduced pressure, each crude peptide was precipitated and washed with ice-cold diethyl ether, then dissolved in 50% aqueous acetonitrile containing 0.1% TFA, and lyophilized.

DNP removal

Crude histidine-containing peptides (2 mM) in 10 ml 6 M guanidine HCl, 200 mM Na\textsubscript{2}HPO\textsubscript{4} buffer, were treated with 200 mM sodium 2-mercaptoethanesulphonate at pH 7.0 for 1 h to remove the DNP-protecting group before purification by reverse-phase HPLC.

Reverse-phase HPLC and LC-MS analysis

Analytical reverse-phase HPLC and LC-MS were performed using an Agilent 1100 series HPLC system equipped with an online MSD ion trap. The column used was Phenomenex Aeris WIDEPORE 3.6-μm C4, 150 × 4.6 mm. Chromatographic separations were obtained using a linear gradient of 5–30% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 25 min, with column temperature 40 °C. Flow rates were controlled at 0.9 ml/min. Peptide detection was based on UV absorption at 214 nm, and masses were obtained by online electrospray mass spectrometry.

Preparative HPLC

Peptide products from solid phase peptide synthesis or from ligation were purified using a Phenomenex Jupiter 5.0-μm C4, 250 × 10.0 mm column. A shallow gradient of acetonitrile (with 0.08% TFA) versus water (with 0.1% TFA) was designed for each peptide based on its elution characteristics. Flow rates were controlled at 5 ml/min. Fractions containing the desired pure peptide were identified by analytical LC and mass spectrometry, combined, and lyophilized.

Native chemical ligation reactions to generate ShK polypeptide analogs

Allo-ShK polypeptides were prepared by native chemical ligation following the same procedure used for the synthesis of L-ShK polypeptide, as described previously (8).

NMR spectroscopy

The sample for NMR was prepared by dissolving lyophilized authentic d-ShK protein (5) in 95% H\textsubscript{2}O, 5% D\textsubscript{2}O at pH 4.9. One-dimensional \textsuperscript{1}H and two-dimensional homonuclear TOCSY (spin lock time, 70 ms) and NOESY (mixing time, 200 ms) spectra were acquired at 20 °C on a Bruker Avance\textsuperscript{3} 600 MHz spectrometer. All spectra were processed in TopSpin (version 3.2; Bruker Biospin) and analyzed using CcpNmr analysis (version 2.1.5). \textsuperscript{1}H chemical shifts were referenced to the 1,4-dioxane signal at 3.75 ppm. Chemical shift assignments for backbone and side-chain protons of d-ShK were made by conventional analysis of two-dimensional TOCSY and NOESY spectra. A complete assignment of the proton NMR signals of d-ShK was obtained.

Author contributions—M. W. P. prepared the original d-allo-ShK. S. C. and R. S. N. recorded and analyzed NMR spectra for d-ShK. S. B. H. K. directed the design and synthesis of the allo-ShK polypeptide analogs. B. D. carried out all the reported syntheses and folding studies. B. D. and S. B. H. K. wrote the manuscript with contributions from all authors.

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