Antiviral Cystine Knot α-Amylase Inhibitors from Alstonia scholaris*

Cystine knot α-amylase inhibitors are cysteine-rich, proline-rich peptides found in the Amaranthaceae and Apocynaceae plant species. They are characterized by a pseudocyclic backbone with two to four prolines and three disulfides arranged in a knotted motif. Similar to other knottins, cystine knot α-amylase inhibitors are highly resistant to degradation by heat and protease treatments. Thus far, only the α-amylase inhibition activity has been described for members of this family. Here, we show that cystine knot α-amylase inhibitors named alstotides discovered from the Alstonia scholaris plant of the Apocynaceae family display antiviral activity. The alstotides (As1–As4) were characterized by both proteomic and genomic methods. All four alstotides are novel, heat-stable and enzyme-stable and contain 30 residues. NMR determination of As1 and As4 structures reveals their conserved structural fold and the presence of one or more cis-proline bonds, characteristics shared by other cystine knot α-amylase inhibitors. Genomic analysis showed that they contain a three-domain precursor, an arrangement common to other knottins. We also showed that alstotides are antiviral and cell-permeable to inhibit the early phase of infectious bronchitis virus and Dengue infection, in addition to their ability to inhibit α-amylase. Taken together, our results expand membership of cystine knot α-amylase inhibitors in the Apocynaceae family and their bioactivity, functional promiscuity that could be exploited as leads in developing therapeutics.

Cystine knot α-amylase inhibitors (CKAIs) are plant knottins with α-amylase inhibition activity. CKAIs typically contain 30–32 residues, including 6 cysteines and 2–4 prolines (1). According to Svensson’s classification, they are the smallest of the seven proteinaceous α-amylase inhibitor families (2). As a group, they have gained increasing attention for their potential as tools for therapeutic treatment and crop protection.

The six cysteine residues in CKAIs are connected by three disulfide bonds in a cystine knot arrangement. To form this structural motif, the embedded ring comprising two of the disulfide linkages and the connecting intercysteinyl loops is threaded by the third disulfide bond. Such a knotted structure provides multiple strand cross-bracings, which give rise to the high tolerance to heat and endoproteolytic treatments of CKAIs and other similar types of plant cystine knot peptides (1, 3, 4). CKAIs are also pseudocyclic peptides that have the disulfide-locked cysteine residues located at the N terminus and the penultimate C terminus, protecting the peptide chain from degradation by exopeptidases (1).

Thus far, nine members of CKAIs from the Amaranthaceae and Apocynaceae families have been characterized. The prototypic CKA1, the Amaranth α-amylase inhibitor AAI, was discovered in 1994 when Chagolla-Lopez et al. (5) identified an α-amylase inhibitor in the crop plant Amaranthus hypochondriacus of the Amaranthaceae family. AAI remained as the only representative member of CKA1 family in the following 20 years. Very recently, we identified and characterized eight CKAIs from apocynaceous plants, including wrightides Wr-A11 to Wr-A13 from Wrightia religiosa and allotides Ac1–Ac5 from Allamanda cathartica (1, 6).

Sequence analysis of nine CKAIs revealed a conserved cysteine spacing of CXc–CXc–CXc–CXc–CXc–CXc, where X represents a non-cysteine residue. Because Cys III and Cys IV are sequentially adjacent to each other in all of the CKAIs identified so far, we have named four intercysteinyl loops in their structures as loops 1–4. Loop 2 of AAI is two or three residues longer than loop 2 of allotides or wrightides, respectively. In addition, the six cysteine residues, Pro in loop 3, and a Gly in loop 4 are conserved in all nine CKAIs.

The structures of three CKAIs have been determined, including the x-ray structure of Wr-A11 and the NMR structures of AAI, Wr-A11, and Ac4 (1, 6, 7). These structures are well defined in solution and highly conserved. They contain three short antiparallel β-strands connected by an optional 310 helix turn in loop 2 and several β-turns. In these structures, the conserved proline in loop 3 exists in cis-configuration. In addition to this conserved cis-proline in loop 3, CKAIs may contain an additional cis-proline in loop 2 or loop 4, making the CKAIs a cis-proline-rich peptidyl family.

An early study on AAI in complex with the Tenebrio molitor α-amylase (TMA) suggested that the Arg7 in loop 1 of AAI plays a critical role in the TMA inhibition and forms the only electrostatic interaction with the catalytic residue Asp287 (TMA) in the TMA:AAI crystal (8). An Arg or Lys at the conserved posi-

* This work was supported in part by Competitive Research Grant NRF-CRP8 – 2011-05 from the National Research Foundation in Singapore. The authors declare that they have no conflicts of interest with the contents of this article.

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2 The abbreviations used are: CKA1: cystine knot α-amylase inhibitor; AAI, amaranth α-amylase inhibitor; TMA, T. molitor α-amylase; CK, cystine knot; UPLC, ultra performance liquid chromatography; NEM, N-ethylmaleimide; TOCSY, total correlation spectroscopy; RACE, rapid amplification of cDNA ends; IBV, infectious bronchitis virus; hpi, h postinfection; DENV2, Dengue type 2; RSV A, respiratory syncytial virus type A; MOI, multiplicity of infection.
tion with Arg7 of AAI is also found in Ac1–Ac7 sequences. However, a positively charged residue at equivalent position is not present in Wr-Al1, Wr-Al2, or Wr-Al3. Our study using a dynamically simulated model of Wr-Al1 and TMA suggested that CKAIs interact with TMA via different molecular networks (1). The unresolved inhibitory mechanisms among CKAIs provide an impetus for identifying additional CKAIs from other plant species to study their inhibitory mechanisms against TMA.

In a program to identify and characterize cysteine-rich peptides as putative active compounds in medicinal herbs, we have profiled systematically their occurrence using mass spectrometry and found cysteine-rich peptides in medicinal plants including apocynaceous plants. *Alstonia scholaris* Linn. R. Br. of the Apocynaceae family is an evergreen tree found in the tropics, and different plant parts have been used as a folklore remedy for a wide spectrum of ailments, ranging from fever, diarrhea, and leprosy to malaria and cancer (9).

Here, we report the discovery and characterization of four novel CKAIs, allostotides S1–S4 hereafter named as As1–As4, from the aqueous extract of the *A. scholaris* plant by both proteomic and genomic methods. We confirmed the cystine knot motif of the As3 by a top-down mapping method using a high temperature partial reduction followed by proteomic analysis. NMR structures of As1 and As4 showed that they have two cis-proline residues. Genomic analysis revealed a three-domain precursor organization of alstotides, a precursor arrangement found previously for CKAIs and other plant knottins. Most interestingly, we show for the first time their membrane permeability and antiviral activity toward infectious bronchitis virus (IBV) and Dengue virus. Molecular studies suggest that As1 is an early acting antiviral peptide and binds to IBV spike (S) and membrane (M) proteins. Together, this work furthers the understanding of the molecular, biological, and structural characteristics of CKAIs and provides insights for developing CKAIs as a potentially useful cell-penetrable scaffold for peptidyl therapeutics.

**Experimental Procedures**

* Purification and de Novo Sequencing of CK α-Amylase Inhibitors—The CK α-amylase inhibitors from *A. scholaris* leaves were extracted in 50% EtOH, purified by HPLC, and sequenced by MALDI-TOF/TOF-MS as described previously for allotides from *A. cathartica* (6). Amino acid analysis was performed as described in the same paper to clarify ambiguity by isobaric residues for As3.

* Thermal and Enzymatic Stability Tests—* Purified peptides were heated to 100 °C for 1 h or treated with chymotrypsin for 0.5, 4, or 24 h. UPLC and MS analysis were performed to examine any sample degradation. Untreated or reduced peptides were used as controls.

* Disulfide Mapping by Partial Reduction and Stepwise Alkylation—* Approximately 0.3 mg of As3 dissolved in 0.5 ml of 30% acetonitrile/H2O and 2-fold volume of 40 mM tris-(2-carboxyethyl)phosphine in 200 mM citrate buffer, pH 3, were incubated at 75 °C for 3.5 min. All reagents were equilibrated to the reaction temperature before mixing. The partial reduction was quenched by immediate injection onto analytical reversed phase HPLC column and eluted with shallow gradient focusing the 38–40% fraction in 30 min.

Alkylation of reduced cysteine residues was carried out by adding an excess amount of N-ethylmaleimide (NEM) directly to the HPLC fraction and incubating the mixture at 45 °C for 45 min. Different NEM-alkylated species were then separated by reversed phase HPLC focusing the 38.5–40% fraction in 1 h. MS scanning of the eluted fractions showed that we obtained As3 alkylated with two to six NEM groups.

For complete reduction and alkylation, fractions that contained As3 alkylated with two/three and three/four/five NEM groups, respectively, were added with equal volume of 40 mM DTT in 25 mM NH4HCO3 buffer, pH 7.8. After an incubation at 70 °C for 15 min, 1/5 volume of 0.5 M iodoacetamide in water was added, and the reaction was allowed for 2 h at 37 °C before injection into reversed phase HPLC.

To prepare the sample for MS/MS sequencing, collected fractions were lyophilized, dissolved in 50 μl of NH4HCO3, and digested with chymotrypsin for 5 min at room temperature. Sequences of chymotryptic fragments were determined based on both b- and y-ion series in the tandem mass spectrometry profiles.

* NMR Spectroscopy—* The NMR sample contains purified As1/As4 in 95% H2O, 5% D2O or 99.9% D2O (~1 mM peptide and pH/pD = 3.3). All NMR experiments were carried out on a Bruker 600-MHz NMR spectrometer equipped with a cryogenic probe. Two-dimensional TOCSY and NOESY experiments were performed with mixing times of 80 and 200 ms, respectively (10). Two-dimensional data were acquired at 298 K. Water suppression was achieved using modified WATERGATE pulse sequences (11). The NMR spectra were processed with NMRPipe software (12). The amides involved in hydrogen bonding were identified by the hydrogen-deuterium exchange one-dimensional 1H experiment (12).

* Resonance Assignment—* Sequence specific assignments were achieved based on the two-dimensional TOCSY and NOESY, and NOEs were assigned from the two-dimensional NOESY, using the software NMRspY. The chemical shifts were deposited in BioMagResBank with accession numbers of 19847 for As1 and 19846 for As4. Distance restraints were derived from the peak intensities of the assigned NOEs. Dihedral angles $\Phi$ were obtained from $J_{HN-Hδ}$ coupling constants measured from the one-dimensional 1H spectrum. Hydrogen bond restraints were incorporated based on the observation of amide protons in the one-dimensional 1H spectra recorded after resuspending the lyophilized As1/As4 in D2O for up to 18 h at 25 °C.

* Structure Calculation—* Structure was calculated with simulated annealing approach with CYANA 2.0 (13). Distance restraints are divided into three classes: 1.8 < $d$ ≤ 3.4 Å (strong NOEs), 1.8 < $d$ ≤ 4.2 Å (medium NOEs), and 1.8 < $d$ ≤ 5.5 Å (weak NOEs). Disulfide bond restraints of 2.0 ≤ $d$ (S$i$, S$j$) ≤ 2.1 Å, 3.0 ≤ $d$ (C$i$, S$j$) ≤ 3.1 Å, and 3.0 ≤ $d$ (S$i$, C$j$) ≤ 3.1 Å were employed for structure calculation. During the structure calculation, hydrogen bond restraints of 1.8–2.2 Å for the $H$-O distance and 2.2–3.2 Å for the N-O distance were applied on nine identified hydrogen bonds according to the slowly exchanging amide protons. $\Phi$ angles were constrained to the range of $-150^\circ$ to $-90^\circ$ for $J_{HN-Hδ}$ > 8 Hz. Structures are dis-
played and analyzed using software PyMOL and program PROCHECK-NMR (14), respectively.

α-Amylase Inhibitor Gene Cloning—Total RNA extraction of the flower was performed using the Purelink Micro-to-Midi kit (Invitrogen) followed by 3′ RACE cDNA synthesis. Targeted genes were amplified in PCRs using a degenerate primer (5′-ATTAACTCaTGyTGyGAr CCnTA-3′) encoding INQCCDPY sequence. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced by 1st Base Company (Singapore). Gene-specific primer (5′-CCTAAATAAAGGTCGCTGTCGCAAG-3′) against the 3′-untranslated regions of the partial genes obtained was designed for PCRs on 5′ RACE cDNA template prepared using the SMARTer RACE cDNA amplification kit (Clontech, Takara, Biotechnology, Dalian, People’s Republic of China). The full-length sequences were obtained by assembling the 5′- and 3′-end fragments. Endoplasmic reticulum signal regions were predicted using the SignalP 3.0 website (15, 16) and utilized to design the forward primer 5′-TGGCTAAGCCTTGCTGTTCACTCCT-3′. This primer was used to randomly amplify related peptides as well as to study their DNA sequences. Genomic DNA extract was prepared from fresh A. scholaris leaves using the PureLink Plant Total DNA purification kit (Invitrogen).

α-Amylase Activity Assays—In this study, we used TMA prepared in-house at pH 5.4 by ammonium sulfate precipitation, anion exchange chromatography, and dialysis as previously described (1). Amylase activity of the enzyme and inhibitory effects of the purified peptides were assayed following the Bernfeld method in 96-well plate (17). The assay was performed essentially as reported previously (1).

Antiviral Activity Assay—Vero cells (4 × 10⁴ cells/well) were seeded and cultured overnight to reach 80% confluence. The cells were incubated with IBV (MOI = 1) for 1 h after the virus was treated with As1 or As3 (25, 50, 75, and 100 μM) for 1 h at room temperature. The virus stock without prior treatment was added to the negative control. The Vero cell monolayers were collected at 24 h postinfection (hpi), lysed with Laemmli sample buffer containing 20 mM DTT and analyzed by Western blotting. IBV nucleocapsid (N) protein and β-actin were probed with their primary antibody and subsequently detected with horseradish peroxidase-conjugated secondary antibody and a chemiluminescent substrate (Amersham Biosciences ECL Western blotting detection reagents; GE Healthcare). The supernatant in the experiment was also collected for the plaque assay. Time course experiments were done similarly for peptides at 50 μM. The samples were collected at 6-h intervals within the first 24 h.

Antiviral assay for As1 against Dengue type 2 (DENV2) and respiratory syncytial virus type A (RSV A) infection was essentially done in a similar manner. The cultures were incubated for 2 (RSV A) to 3 (DENV2) days until >50% cytopathic effect appeared. The cells treated with virus only served as the negative control. Anti-RSV antibody was purchased from Abcam (ab43812). Antibody against DENV2 NS3 was prepared in-house (18).

Time of Drug Addition Assay—Vero cells seeded in wells labeled A–E were incubated with IBV (MOI = 1) at 4 °C for 2 h to facilitate viral attachment. As1 (50 and 100 μM) was added together with the virus for sample A before the incubation. After the 4 °C incubation, the cells were washed once with PBS to remove unbound virus particles, and the medium was replaced with new DMEM before the cells were transferred to 37 °C. At the transfer or 3, 6, and 8 hpi, As1 was added to parallel cultures at the final concentration of 50 and 100 μM. Equal amounts of peptide were also dispensed to sample A to replace the washed out peptide. At 24 hpi, the cell monolayers were collected for Western blotting against IBV N and S proteins. IBV-infected Vero cells without peptide treatment served as the negative control.

Transfection Assay—Subconfluent Vero cells grown on 100-mm dishes were trypsinized, washed twice with cold DMEM, and resuspended in 1.2 ml of ice-cold PBS. Vero suspension (400 μl) was transferred to a prechilled electroporation cuvette together with 2 μg of viral RNA (in up to 5 μl). Electroporation was performed with one pulse at 450 V and 50 microfarads and infinitive resistance using the Gene Pulser II electroporation system (Bio-Rad). After electroporation, the cells were left on ice for 1 min, resuspended in prewarmed complete media, and plated in 24-well plates for Western blotting (500 μl/well) and 12-well plates for quantitative PCRs (1,000 μl/well), after which As1 (100 μM) was added. The cells were incubated at 37 °C, 5% CO₂ and harvested after 3, 18, and 36 h post-transfection. The samples collected at 3 h after seeding served well as the starting control to calculate the percentage replication of IBV virus.

Reverse Transcription and Real Time PCR Analysis—Total RNA of the monolayers was extracted using TRIzol reagent following the manufacturer’s instruction. First strand cDNA library was prepared using the Transcriptor first strand cDNA synthesis kit (Roche Applied Science) according to the manufacturer’s protocol with either oligo(dT)ₐₙ or IBV3835-F (5′-GGAGACAAACACTTGCATG-3′). Primers used in real time PCRs include GAPDH forward (5′-GGAGCCGATGCCCTC-CAAAAT-3′) and GAPDH reverse (5′-GGCTGTGTGCTCAT- ACT-TCTCATG-3′) for the GAPDH gene and IBV3835 forward (5′-GGAGACAAACACTTGCATG-3′) and IBV4321 reverse (5′-TCGCGCCACTCTAGTATAAG-3′) for IBV positive-stranded genomic RNA. Quantitative real time PCRs were performed using 1 μl of cDNA and Power SYBR Green PCR 2× Master Mix on the 7500 real time PCR system (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: 1 cycle of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction was done in triplicate. The data were analyzed with the 7500 software (Applied Biosystems).

Preparation of Biotinylated Peptide—The biotinylation of As1 was performed based on the efficient reaction of N-hydroxysuccinimide-activated biotin with primary amine at pH 7–9 to form stable amide bonds. Biotin reagent was stored at 4 °C and equilibrated to room temperature prior to the reaction to avoid moisture condensation. Biotin in dimethyl sulfoxide (6.8 mg/ml) was mixed with an equal volume of As1 in PBS, pH 7.4 (1 mg/ml), and incubated at room temperature for 30 min. The products were monitored by MS and purified by analytical reversed phase HPLC. Eluted fractions containing biotinylated...
As1, hereafter denoted as biot-As1, were collected, lyophilized, and dissolved in water prior to storage at 
20 °C.

In Vitro Pulldown Assay—Biot-As1 (20 g) was incubated with 30 l of NeutrAvidin UltraLink beads (Thermo Scientific) for 30 min at room temperature with rocking. The beads were washed twice with PBS before incubation with the IBV-infected cell lysates for 2 h at 4 °C with rocking. Unbound proteins and unspecific binding proteins were removed later by two washes with lysis buffer and two washes with SNNTE buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5% sucrose, 1% Nonidet P-40, and 0.5 M NaCl; 1 mM DTT and protease inhibitors added before use). Protein complexes were eluted with Laemmli sample buffer, and the protein complexes were eluted with Laemmli sample buffer and analyzed by Western blotting. Pulldown assay for the biotinylated control peptide with random sequence (biot-KALVINSV) was done in a similar manner. Biot-KALVINSV peptide was prepared in-house using Fmoc (N-(9-fluorenyl)-methoxycarbonyl) chemistry.

Co-immunoprecipitation Assay—Vero cells were infected with IBV (MOI = 5) and co-treated with As1 (75 lM) for 24 h. The lysate of infected, As1-treated cells was incubated with NeutraAvidin beads for 30 min at room temperature with rocking. The beads were subsequently washed thrice with lysis buffer, and the protein complexes were eluted with Laemmli sample buffer. Western blotting was performed to analyze As1-binding M proteins.

Immunofluorescence Assay—Vero cells seeded at a density of 6 × 10^4 in 4-well chamber slides were cultured overnight and inoculated with either DMEM alone, IBV (MOI = 1), biot-As1-treated (10 lM) IBV, or biot-As1 (10 lM) for 16 h at 37 °C until the cytopathic effect was observed for 70–80% of the cell population. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and subsequently blocked with blocking buffer (5% FBS in PBS) before incubation with rabbit anti-IBV-N protein antibody at 4 °C for 2 h. Secondary antibody was goat anti-rabbit immunoglobulin conjugated with Alexa Fluor 594 (dilution 1:200; Life Technologies). After washes with PBS, the cells were stained with NeutrAvidin Dylight 488 conjugate (dilution 1:200, Thermo Scientific). The slide was mounted in Vectashield with counterstain DAPI (Vecto Labs). The fluorescence was examined on a Zeiss LSM 710 META confocal microscope at a magnification of ×63.

Results

Purification and de Novo Sequencing of CKAIs from A. scholaris—To gain insights into sequence variation of CKAIs and its implication in structure and function, we characterized CKAIs expressed in the medicinal A. scholaris plant. In our mass spectrometry screening, we found clusters of peptides with molecular weights of 3000–5000 in A. scholaris leaf, flower, fruit, and stem (Fig. 1), which contain six cysteine residues as revealed by reductive S-alkylation. The peptide expression profiles in different tissues varied relative abundance, but the major peaks (m/z 3250, 3317, 3366, and 3385) were found in all tested plant parts. We next carried out aqueous ethanol extraction combined with multiple dimensions of HPLC purification monitored by MS analysis to obtain purified samples for further characterization. Four predominant peptides, named alstotides S1–S4, or As1–As4, were obtained in a scale-up experiment (1 kg of fresh leaves) with the yield ranging from 3 mg (As2) to 40 mg (As1). Each purified alstotide was disulfide-reduced and sequenced by tandem MS/MS as described previously (Table 1). Ambiguities by isobaric residues in MS/MS de novo sequencing were clarified by either gene cloning (As1, As2, and As4) or amino acid analysis (As3).

All four alstotides contain 30 residues, of which 19 are absolutely conserved. The cysteine pattern and spacing of alstotides resemble the CC-type CK motif found in other CKAIs. They are rich in cysteine (6 residues), proline (4 residues), and glycine (3 residues). As1 and As2 are nearly identical with only one-residue difference: His20 in As1 is replaced by Arg20 in As2. Among CKAIs, alstotides exhibit the highest sequence homology with...
allotides, particularly in loops 2–4 with 11 of 17 intercysteinyl residues conserved (6).

The four allotides As1–As4, together with the nine reported CKAIs, provide information on sequence diversity of CKAI family. Sequence comparison showed that they exhibit extensive variation among sequences from different phylogenetic families. As illustrated by the sequence logo, besides the six cysteine residues, only four residues are found to be conserved between AAI and other apocynaceous CKAIs. They include Pro and an aromatic residue following Pro in loop 3, a side chain OH-containing residue (Thr/Ser) at the beginning and Gly near the end of loop 4. One positively charged residue (Lys/Arg) located at the end of loop 1 was also found in 9 of 13 known CKAIs. A higher sequence homology was observed among CKAIs from the Apocynaceae family. In addition to the residues conserved to AAI, allotides, allotides, and wrightides also share Gln13 in loop 2 and Pro24 in loop 4. Interestingly, proline was found in loops 1–3 of AAI, whereas it is distributed in loops 1, 3, and 4 of apocynaceous CKAI sequences.

Thermal and Proteolytic Stability—Heat treatment and proteolysis generally lead to significant loss of bioactivity of peptides and proteins in herbal remedies. Previously we showed that CKAIs are resistant to both heat and proteolytic degradation (1, 6). To investigate how tolerant these stabilities are to sequence variation, we heated As1 and As3 in boiling water for 1 h. The treated samples and the untreated controls were subjected to UPLC. B, proteolytic stability of As4. As4 was digested by chymotrypsin for different time intervals, and the products were monitored with UPLC and MS. Reduced As4 was treated in similar conditions to serve as a control.

| Plant CKAIs from Amaranthaceae and Apocynaceae families | Species | Peptide | Sequence | Charge | References |
|----------------------------------------------------------|--------|---------|----------|--------|------------|
| **Amaranthaceae family**                                  | A. hypochondriacus | AAI     | CIPKNW-RGPKMDGVPCCETYCTSDYYG-NCS | 0      | (5)        |
| **Apocynaceae family**                                   | W. religiosa   | Wr-AI1  | CAQKGEY-C-SVYL--QCCDPYHTQPVIGGICA | 0      | (1)        |
|                                                          | Wr-AI2  | CAQKGEY-C-SVYL--QCCPKYPQCTQPVIGGICA | +1     | (1)        |
|                                                          | Wr-AI3  | CAQKGEY-C-SVYL--QCCPKYPCTQPVIGGICA | +2     | (1)        |
|                                                          | A. cathartica | Ac1     | CIAHYG-KCDGIIN--QCCDPWLTTPPIIG-ICIC | 0      | (6)        |
|                                                          | Ac2     | CRP-VGRCDGVIN--QCCDPYWTTPPIYG-WCK  | +1     | (6)        |
|                                                          | Ac3     | CRP-YGTRCDGVIN--QCCDPYWTTPPIYG-WCK  | +1     | (6)        |
|                                                          | Ac4     | CIAHYG-KCDGIIN--QCCDPWLTTPPIIG-FCL  | 0      | (6)        |
|                                                          | Ac5     | CVSHYG-KCDGIIN--QCCDPWLTTPPIIG-FCL  | 0      | (6)        |
|                                                          | A. scholaris | As1     | CRP-YGRCDGVIN--QCCDPYHTTPPIIG-ICL  | +1     | This work  |
|                                                          | As2     | CRP-YGRCDGVIN--QCCDPYCTTPPLIG-ICL  | +1     | This work  |
|                                                          | As3     | CVPRFG-RCDGIIN--QCCDPYLTTPPLVIG-ICT | 0      | This work  |
|                                                          | As4     | CVPQYGV-CGDIIN--QCCDPYCSPPPIYG-HCI | -1     | This work  |

FiguRe 2. Proteolytic and thermal stability of allotides. A, heat stability of As1 and As3. The peptides were dissolved in water and heated in boiling water for 1 h. The treated samples and the untreated controls were subjected to UPLC. B, proteolytic stability of As4. As4 was digested by chymotrypsin for different time intervals, and the products were monitored with UPLC and MS. Reduced As4 was treated in similar conditions to serve as a control.
ment. In enzymatic stability assay, >95% of As4 remained intact after a 4-h treatment with chymotrypsin, whereas the reduced As4 control was largely digested into chymotryptic fragments within 5 min.

**Disulfide Mapping of Alstotides**—To determine the disulfide connectivity in alstotides, we performed a top-down mapping method using differential S-reduction and S-alkylation to tag Cys residues by NEM and iodoacetamide. Though highly homologous to Ac2, partial reduction of As3 at 37 °C in various reducing conditions did not yield significant amounts of intermediates as obtained for Ac2 (6). Thus, we employed a high temperature partial reduction by tris(2-carboxyethyl)phosphate at 75 °C for 3.5 min, immediately followed by HPLC purification. Intermediates with one or two disulfide bonds were labeled 1SS and 2SS, respectively. The free cysteinyl thiols of these intermediate species were S-alkylated with excess NEM. The mass shifts in MALDI-TOF MS of S-alkylated species were used to determine the number of NEM groups in each species. Each NEM-labeling caused a mass increase of 126 Da.

The 1SS and 2SS species were later fully reduced with DTT and the newly reduced half-cystine alkylated with iodoacetamide. Each differentially labeled species was digested by chymotrypsin and sequenced by tandem MS/MS (Fig. 3). The analysis of 2SS species with two NEM tags on Cys I and Cys IV revealed a disulfide bond between these two cysteine residues. Likewise, a disulfide linkage between Cys III and Cys VI was implicated by the positions of two iodoacetamide groups in 1

**NMR Structure of Alstotides**—The solution structures of As1 and As4 (Fig. 4) were determined using the restraints on distance, dihedral angle, and hydrogen bonds derived from TOCSY, NOESY, and hydrogen/deuterium exchange 1H experiments (Table 2). The average root mean square deviations for As1 and As4 are 0.77 ± 0.15 and 0.73 ± 0.17 Å for all backbone atoms, and 1.17 ± 0.18 and 1.22 ± 0.21 Å for all heavy atoms, respectively. Structure alignment revealed a high structural similarity between As1 and As4 with a cystine knot fold: the six cysteine residues are bridged in the order of Cys I–IV, Cys II–V, and Cys III–VI, where Cys III–VI is the penetrating linkage, confirming our results by the chemical analysis of As3 using a differential labeling method. A highly twisted β-sheet formed by three short β-strands (residues 6–8, 20–22, and 27–30) and connecting β-turns was identified in both structures. The most significant backbone differences between As1 and As4 structures occur in loops 2 and 4 (Ile11–Asn13 and Pro23–Pro24, respectively, As1 numbering) as shown in Fig. 4C. These residues have previously been implicated to play negligible role in the interaction of CKAI and TMA (1). Analysis of hydrogen/deuterium exchange showed slow exchange rates for 25–40% amide protons in As1 and As4 form stable intramolecular hydrogen bonds. Both As1 and As4 contain four proline residues located in loops 1, 3, and 4. The configurations of proline in both peptides were unambiguously identified as trans-Pro3, cis-Pro18, cis-Pro23, and trans-Pro24, based on the presence of NOE cross-peaks of Hα Res(i-1)–Hβ Pro(i) for cis-configurations and Hα Res(i-1)–Hβ Pro(i) for trans-configurations.

**Gene-encoded Alstotide Precursors**—Cysteine-rich peptides, such as cyclotides, are encoded by genes with diverse precursor structures (3, 19, 20). Previously we showed that allotides and wrightides contain three domains in their precursors (1, 6). To determine whether alstotide genes share the same organization with other CKAI, we isolated and sequenced alstotide clones. A degenerate primer targeting the conserved INQCCDPY motif was used in 3′ RACE PCR, from which the partial sequence of the gene encoding As1 was obtained. We next designed a specific primer against the 3′-untranslated region of this partial gene for 5′ RACE PCR. The reaction yielded the complete transcript of As1 precursor, named asc1. A specific primer against the signal peptide of asc1 clone was subsequently used to amplify asc2 and asc4 clones of As2 and As4, respectively, on DNA template. The gene sequences of asc1, asc2, and asc4 were deposited in the GenBank under the accession numbers KP318736, KP318737, and KP318738, respectively.

The three precursors encoded by asc1, asc2, and asc4 were highly homologous to each other and to allotide and wrightide clones (Fig. 5). They are 88–92 residues long and contain three domains: a 21–22-residue signal peptide targeting endoplasmic reticulum, a 36–41-residue pro-domain, and a 30-residue mature peptide followed immediately by a stop codon. We also found a phase 1 intron of 135 and 192 base pairs in the middle of the signal peptide of asc2 and asc4, respectively. Sequence alignment of all CKAI precursors known so far showed that their signal peptide and the mature peptide are highly conserved, whereas the pro-domain is variable in size. The pro-domain ranges from 36 residues in asc4 clone to 51 residues in asc1, asc4, and asc5.

**α-Amylase Inhibitory Activity of Alstotides**—The α-amylase inhibitory effect of alstotides was studied on α-amylase from *T. molitor* larvae (TMA), human salivary, and fungus (*Aspergillus oryzae*). TMA was known to be inhibited by AAI, wrightides, and allotides (1, 5, 6).

Activities of TMA before and after incubation with As1, As3, and As4 were measured by Bernfeld method. The results showed that all three alstotides had inhibitory effects toward TMA (Fig. 6A) with IC50 values in the range of 1.9–5.2 μM. Similar to other CKAI, all three alstotides did not inhibit human and fungal α-amylases at up to 100 μM.

**Antiviral Activities of Alstotides**—Cytotoxicity assay showed that As1 and As3 did not induce significant cytotoxicity in Vero cells at concentrations up to 100 μM. Thus, subsequent assays were conducted with peptide concentrations in the range of 0–100 μM.

IBV is a γ coronavirus and the causative agent of infectious bronchitis, a highly infectious respiratory disease in fowl, particularly young chickens, causing a major economic loss in the poultry industry worldwide. To evaluate the ability of alstotides to inhibit IBV replication, antiviral assay was performed using a pretreatment protocol. Briefly, virus stocks were pretreated with 25, 50, 75, and 100 μM of peptides, respectively. Vero cells were inoculated with untreated or peptide-treated IBV at MOI = 1, and viral replication was evaluated at 24 hpi (Fig. 6, B and D). IBV replication in the absence of alstotides was scored 100%, which was used to normalize the treated samples. The

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FIGURE 3. Tandem MS/MS profiles for disulfide connectivity determination of As3. The MS/MS profiles of tryptic fragments of alkylated 1SS (A) and 2SS (B) intermediates. The disulfide connectivity was confirmed to be Cys I–IV, Cys II–V, and Cys III–VI.
results showed that pretreatment with As1 and As3 could inhibit plaque formation in a dose-dependent manner with an estimated EC_{50} of 35 and 55 μM, respectively. The inhibitory effects of these peptides at MOI = 1 were further visualized in the time course assay (Fig. 6, C and E). We also found that the EC_{50} value is dependent on virus concentration. EC_{50} of As1 decreases to 6 and 4 μM as MOI decreases to 0.1 and 0.05, respectively. In an antiviral assay (Fig. 7F), biot-As1 was shown to partially retain the inhibition activity against IBV of As1 at 50 μM (38% inhibition as compared with 92%) and to lose most of antiviral activity at 10 μM (5% inhibition as compared with 55%). Because As1 showed a stronger antiviral activity, As1 was chosen as the model to study the antiviral activities and the underlying mechanisms in subsequent experiments.

With a need to find new drugs targeting human viral diseases and also to investigate the antiviral specificity of alstotides, As1 was tested against DENV2 and RSV A. The effect of As1 on these two viruses was evaluated in a co-treatment antiviral assay (Fig. 6, F and G). As1 (25–100 μM) was added to the cell culture at the point of viral infection, and the cell lysate was
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| Plant cystine knot peptides | Signal Peptide | Pro-domain | Cystine knot peptide |
|----------------------------|----------------|------------|---------------------|
| α-amylase inhibitor        | A. scholaris   | (21-22)    | (36-41)             | (30)                |
| As1                        | MAKKLAAAATAAA-TENYAA---TYEAKVELEL---         |          |                     |
|                            |                |            |                     |
|                            | As3            | (21-22)    | (28-51)             | (30)                |
|                            | MAKKLAAAATAAA-TENYAA---TYEAKVELEL---         |          |                     |
|                            |                |            |                     |
|                            | A. oenotheraeae| (21-22)    | (38)                |                     |
| Ao1                        | MAKCLAAATAAA-AVRYV---TAEQDIQETVNGXKLGKNVW70HNLHAAINLTLFLEPPERAPA--- |          |                     |
|                            |                |            |                     |
|                            | Ao3            | (21-22)    | (28-39)             |                     |
|                            | MAKCLAAATAAA-AVRYV---TAEQDIQETVNGXKLGKNVW70HNLHAAINLTLFLEPPERAPA--- |          |                     |
|                            |                |            |                     |
|                            | W. religiosa   | (23-26)    | (21-22)             |                     |
| Wr-A11                     | MAKKLAAAATAAA-TENYAA---TYEAKVELEL---         |          |                     |
|                            |                |            |                     |
|                            | Wr-A12                     | (23-26)    | (21-22)             |                     |
|                            | MAKKLAAAATAAA-TENYAA---TYEAKVELEL---         |          |                     |
|                            |                |            |                     |
|                            | S. tuberosum    |             |                     |
| T. kirilowii               | MAKKLAAAATAAA-TENYAA---TYEAKVELEL---         |          |                     |
|                            |                |            |                     |
|                            | T. c.           | (21-22)    | (28-39)             |                     |
|                            | MAKKLAAAATAAA-TENYAA---TYEAKVELEL---         |          |                     |

FIGURE 5. Alignment of deduced amino acid sequences of CKAI precursors and two representative examples of plant knottin precursors. Precursors are comprised of an endoplasmatic reticulum signal sequence, a pro-domain, and a mature cystine knot domain. Stop codons are depicted by asterisks. The numbers in parentheses indicate the size ranges of each domain in each cluster.

harvested after 2 (RSV A) to 3 days (DENV2). The assessment of DENV2 NS3 protein expression in the infected cultures revealed a moderate inhibition of As1 against DENV2. EC50 was estimated to be ~90 μM based on semi-quantitative analysis by densitometry. In contrast, As1 did not inhibit the replication of RSV A at up to 100 μM, as observed from comparable intensities of F0 and F1 protein bands for all infected culture in Western blotting analysis.

The antiviral mechanisms of As1 were studied by using IBV as a model system. We first determined the stage(s) in viral replication cycle which alstotides could inhibit using a time of drug addition assay. As1 (50 and 100 μM) was introduced to synchronized infected cells at different time points during pre-incubation at 4 °C and the infection at 37 °C (Fig. 7B). In this assay, As1 added during attachment (at 4 °C) or entry (after transfer to 37 °C) exhibited comparable positive effects. As1 significantly lost its activity when added during later stages from 3 hpi onward. Comparable intensities of the endogenous control actin bands for both the treated and untreated samples traversed the cell membrane and accumulated intracellularly (Fig. 8).

We performed a transfection assay in which the attachment and entry were bypassed to further confirm that As1 acts mainly during the early stage of IBV replication. In this assay, the cells were transfected with purified viral RNA before treatment with 100 μM of As1 and viral protein expression were quantified at 18 and 40 hpi (Fig. 7C). Quantitative real time PCR for positive-stranded genomic RNA did not show significant difference between the As1-treated sample and the untreated control collected at 18 hpi. In contrast, a remarkable inhibition of As1 on secondary IBV infection was observed at 40 hpi. These results confirmed that biot-As1 forms an intracellular association with M protein in infected Vero cell system. To study the specificity of interactions between biot-As1 and IBV proteins, a pulldown assay using a biotinylated control peptide was performed. The results showed that the control peptide did not pull down S protein, ruling out the possibility that the biotin moiety in biot-As1 nonspecifically pulls down membrane-associated IBV proteins (data not shown).

Cell Permeability of As1—One of the major disadvantages of current therapy using peptides is their low bioavailability because of the limited cell membrane permeability. It is thus of interest to determine the cell permeability of alstotides using As1 as a representative example. Using immunofluorescence staining, Vero cells were treated with biot-As1 in the presence or absence of IBV at 37 °C for 16 h. The mock sample was not treated with either biot-As1 or IBV. The cells were fixed, permeabilized, and stained with NeutrAvidin conjugated to Dylight 488. Confocal microscopy images showed that biot-As1 in both infected and uninfected samples traversed the cell membrane and accumulated intracellularly (Fig. 8).

Discussion

In this work, we report the discovery and characterization of four new members of the CKAI family from the aqueous extract of A. scholaris. As1–As4. With the discovery of four alstotides, A. scholaris becomes the third species from the Apocynaceae family known to express CKAIas. With two residues shorter than AAI, the 30-residue apocynaceous CKAIas are currently the smallest α-amylase inhibitors known to date (1). Using proteomic, genomic, and structural studies, this work furthers our understanding about molecular diversity of CKAIas regarding their sequence, structure, and function. The solution structures of As1 and As4 determined in this study provide insights into the conserved structural features of CKAIas from the Amaranthaceae and Apocynaceae families. We also showed here the membrane permeability of CKAIas and their antiviral activity as an early acting inhibitor against infectious bronchitis virus. The novel antiviral effect discovered for CKAIas in this study sug-
suggests that, similar to other cystine knot peptide families such as cyclotides and defensins, CKAIas possess a promiscuous array of biological functions (21). Thus, it is of interest to explore their therapeutic properties and potential applications as leads in developing peptidyl drugs.

Homologous Precursor Structures of Apocynaceous CK Inhibitors—Genetic characterization reveals a three-domain structure of alstotide precursors: an endoplasmic reticulum signal peptide, a pro-domain, and a single mature peptide domain. A phase 1 intron is present in the middle of the signal peptide in genomic sequences. This precursor organization is previously reported to be adopted by other CKAIas such as wrightides and allotides to encode their genes (1, 6). This gene organization suggests that alstotides are essentially synthesized as larger precursors that will be N-terminally processed by SPase I to remove the signal peptide and then follow the cellular processing pathway for secretory proteins.

Among alstotides, remarkable homology was found between acs1 and acs2 clones, suggesting that alstotides, and perhaps other CKAIas, have evolved from recent gene duplication and mutation events within plant species through a process widely known as evolutionary divergence. The divergent process through duplication and mutation to enrich the gene population of important biomolecules is an efficient mechanism adopted by many plants for better adaptation to environmental challenges and enhanced survival. Such homologous genes in plant multimember protein families have been previously reported. Examples include the 43-kDa α-amylase inhibitors αAI-1 and αAI-2 from the common bean (Phaseolus vulgaris L.) that share 78% sequence homology (22).

The conserved gene structure and high sequence homology of CKAIas within the Apocynaceae family, particularly at signal peptide and functional domain, suggest that the CKAI gene existed in the ancestral Apocynaceae species before differentiation events leading to the emergence of diverse CKAI-producing species. Each domain in the precursor has evolved under the pressure of evolutionary adaptation and survival. The pro-domain, believed to aid in the biosynthesis of mature CKAI domain rather than playing any major function, undergoes significant changes and is the most variable in length and sequence among the three domains. The two other domains, including signal peptide destined to endoplasmic reticulum and CKAI mature peptide probably functioning in plant defense, are more conserved than the pro-domain.

The Importance of High Proline Content and a cis-Proline Bond—The NMR structures of alstotides, using As1 and As4 as representative models, were determined to study structural tolerance of CKAIas to sequence variation. Similar to other cysteine-rich peptide families such as cyclotides and plant defensins (23–25), CKAIas show a high structural tolerance to amino acid sequence variations of intercysteiny1 residues and loop size, both within and between families. Of note among these variations are the sequence and loop length differences in loop 2 of AAI (8 residues) and Wr-AI1 (5 residues), which probably leads to the presence of a short α-helix turn in this loop (Pro10–Asp13) of the amaranthaceous AAI and its absence in the apocynaceous CKAIas. Despite these variations, CKAIas share several conserved features: 1) they all adopt a cystine knot fold; 2) their backbones are well aligned and accommodate three short antiparallel β-strands connected mainly by β-turns, and these β-strands are found in similar location and orientation in the CKAI backbone alignment; and 3) the structures are stabilized by an abundance of stable intramolecular hydrogen bonds that exhibited slow hydrogen/deuterium exchange rates in NMR experiments.

Sequence and structural comparison of structurally resolved CKAIas, including AAI, Wr-AI1, Ac4, As1, and As4, revealed their tendency for cis/trans-configuration of proline residues as summarized in Fig. 4B: a trans-proline in loop 1 (observed for AAI, As1, and As4), cis-proline in loop 3 (all sequences), cis-
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**FIGURE 7.** Anti-IBV mechanism of As1. **A,** the IBV replication cycle. **B,** time of drug addition assay. Parallel cultures of Vero cells with synchronized IBV infections were treated with As1 (50 and 100 μM) at different time points. The cell monolayers were collected at 24 hpi. Densitometry analysis as shown in the table underneath the Western blotting results revealed that all As1-treated samples showed significant reduction in the synthesis of S, M, and N proteins as compared with the control (p < 0.01), except the synthesis of M protein in sample E when treated with 50 μM of As1. C, quantification of virus RNA replication when the entry step was bypassed. IBV RNA was electroporated into two parallel cultures of Vero cells, and As1 (100 μM) was added into one of the cultures. The viral replication was analyzed at 18 hpi (primary infection) and 40 hpi (secondary infection) by Western blotting and real time PCR. PCR quantification of positive-stranded genomic RNA was normalized against that of the 3-hpi sample. **D,** in vitro interaction between As1 and IBV protein using pulldown assay. Viral proteins in IBV-infected cell lysate were incubated with biot-As1 immobilized on NeutrAvidin beads and analyzed by Western blotting. Noninfected cell lysates were used as a control. **E,** co-immunoprecipitation of As1 and M protein. IBV-infected cells were treated with As1 (75 μM) and incubated for 24 h at 37 °C. Cell lysates were precipitated with NeutrAvidin beads and blotted with anti-M protein antibody. Protein markers are shown in kDa. **F,** antiviral effect of biot-As1 against IBV infection at 24 hpi. The experiment was done at MOI = 0.1. Intensity of each band was quantitated using densitometry and normalized to β-actin. Percentage inhibitions of samples treated with As1 and biot-As1 were calculated against negative control, which was scored 0%.

Proline followed by trans-proline in a CXPP motif (observed for Ac4, As1, and As4), or a trans-proline in CXXX motif (for Wr-A11) in loop 4, where X is a non-cysteine residue. As such, the presence of the conserved cis-Pro16 in loop 3 has become a defining feature of all CKAIs so far. This cis-proline is typically stabilized by parallel stacking of its pyrrolidine ring and the aromatic side chain of the following Tyr or Trp residue (distance in the range of 5 Å).

**Antiviral Mechanism of Alstotides**—Worldwide medical challenges of viral infection have fostered efforts in developing antiviral drugs as an alternative treatment to vaccines and antibodies. In addition, emerging resistant viruses also pose a significant challenge. However, despite a number of potent small molecule antiviral substances reported in the past decades, most peptide-derived drugs have been plagued by many unsatisfactory therapeutic characteristics. They generally have poor oral bioavailability, high cytotoxicity, and off target effects. Here we describe the discovery of alstotides as cell-permeable, metabolic-stable, and noncytotoxic peptides with antiviral activities against IBV and DENV2. We also provide in this study insights into the possible antiviral mechanisms of alstotides against IBV infection on Vero cells.

At noncytotoxic concentrations up to 100 μM, As1 was identified as a moderate early acting anti-IBV drug, as supported by three lines of evidence. First, the time of drug addition assay revealed a significant drop of activity when As1 was added at 3
hpi, implicating an antiviral mechanism upstream of genomic replication and gene expression. Second, in transfection assay when the genetic material was electroporated into the host cells to bypass the attachment and entry steps, no inhibition over gene replication within 18 hpi was observed. In contrast, parallel As1-treated samples collected at 40 hpi showed a clear inhibition, probably because of inhibitory activity during the early stage of secondary infection. Third, As1 binds to IBV fusion glycoprotein, S protein, which plays a vital role during viral entry by attaching to Vero cell receptors and triggering membrane fusion between enveloped virus and host cells (26). It is thus possible that the putative antiviral mechanism of As1 is through its interference with S protein function during IBV entry. We do not rule out the possibility that As1 may display off target effects or elicit an immune response in vivo; however, its noncytotoxic nature still makes it a potential candidate for anti-IBV drugs.

Upon biotinylation, biot-As1 exhibited a significant decrease in antiviral activity. We rationalize that blocking of the N-terminal sequence and possibly proximal residues, rather than structural change, was the main reason for the partial loss in cellular permeability of allostotides by indirect immunofluorescence. Vero cells were treated with biot-As1 at the point of infection by IBV and incubated for 16 h. The cells were stained with rabbit anti-IBV N protein sera, followed by FITC-labeled anti-rabbit antibody (green) and then NeutrAvidin conjugated to DyLight 594 (red). The nuclear DNA was visualized with DAPI in the mounting medium (blue). Parallel samples without either As1 treatment (labeled IBV) or IBV infection (labeled As1) or both (mock) were prepared as controls. Magnification is ×63.
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antiviral activity upon biotinylation. First, CKAIs and other cystine knot peptides are known for their remarkably stable structures rigidified by three interlaced disulfide bonds. Hence, it is unlikely that their structure will be significantly changed by a biotin group covalently bonded to the flexible extended N terminus. Second, the biotin moiety was linked to As1 via an aliphatic aminohexanoic acid spacer, which is commonly used to minimize the structural alteration induced by the introduction of a biotin group. Together, the partial loss in anti-IBV effect of biot-As1 suggested that the N-terminal Cys residue and its spatially neighboring residues might be involved with the antiviral mechanism of As1.

In pulldown assays, we also identified specific association of As1 and IBV M protein, which is believed to play a major role in the assembly, budding, and maturation processes (27). M protein is comprised of a small glycosylated ectodomain, a triple membrane-spanning domain, and a large endodomain (28). A preliminary attempt to map this interaction excludes the possibility of As1 binding to the glycosylation group of M protein (data not shown). The As1-M protein association suggests that As1 may elicit weak inhibition on IBV assembly and/or budding process. This hypothesis is supported by a positive effect observed in the time of drug addition assay when As1 was added from 3 hpi onwards. In addition, the confocal images presented showed clearly that biotinylated As1 can gain entry into both infected and noninfected cells. It may interact with IBV proteins during viral protein translation and viral particle assembly, as well as maturation and release. It would be interesting to further study the co-localization of biot-As1 with IBV M protein in a detailed time course experiment. Unfortunately, the polyclonal anti-IBV M antiserum available is not suitable for such a study.

Author Contributions—P. Q. T. N. was mainly responsible for design, data acquisition, data analysis, and manuscript preparation. J. S. G. O. contributed to peptide purification and characterization. N. T. K. N. and M. H. contributed to data acquisition and analysis in antiviral study. S. W. determined the NMR structures. J. P. T. and D. X. L. coordinated the study and wrote the paper.

Acknowledgment—We thank Dr. Samuel Ko (Carl Zeiss Singapore) for technical support on immunofluorescence microscopy.

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