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Synthesis and Stereochemical Assignment of Conioidine A: DNA- and HSA-Binding Studies of the Four Diastereomers

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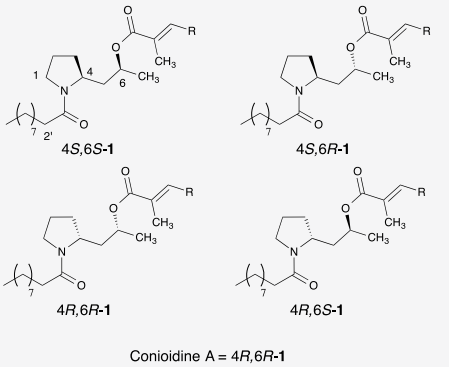
ABSTRACT: Conioidine A (**1**), isolated in 1993 with unknown relative and absolute configuration, was suggested to be a DNA-binding compound by an indirect technique. Four stereoisomers of conioidine A have been synthesized from D- and L-proline, and the natural product has been identified as possessing (4*R*,6*R*) absolute configuration. Binding of the conioidine diastereomers to calf thymus DNA (CT DNA) and human serum albumin (HSA) has been investigated by fluorescence spectroscopy and isothermal titration calorimetry (ITC). All stereoisomers display at least an order of magnitude weaker binding to DNA than the control compound netropsin; however, a strong association with HSA was observed for the (4*R*,6*S*) stereoisomer.



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Conioidines A and B are pyrrolidine natural products isolated from the Texas plant *Chamaesaracha conioides* by Chan and co-workers in 1993.[1] The gross structure of each compound was established by means of 1D and 2D NMR spectroscopy; however, the relative and absolute configurations of both structures were not elucidated (Figure 1). Intriguingly, cytotoxicity assays in the presence and absence of exogenous DNA suggested that both compounds bind DNA with approximately micromolar affinity. Unlike most naturally occurring substances that interact with DNA,[2] the conioidines lack aromatic ring systems that are typically important structural elements for tight binding to nucleic acids. It has been recently demonstrated that leinamycin, a natural product that lacks a polycyclic aromatic unit, binds DNA by intercalation of a (*Z*,*E*)-penta-2,4-dienone moiety in the base pair stack.[3] Furthermore, intercalation of hydrophobic amino acid side chains between base pairs is an important binding element in numerous DNA−protein complexes.[4] To elucidate the absolute configuration of the conioidines and evaluate their DNA-binding profile, we undertook the synthesis of all four diastereomers of conioidine A.

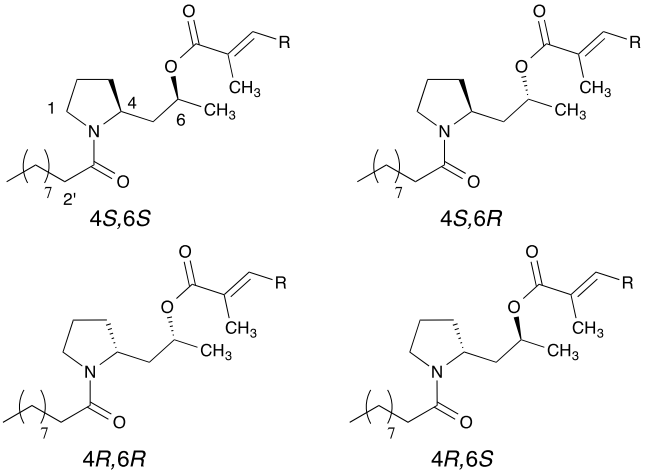

Figure 1. Four possible diastereomers of conioidines A and B.

RESULTS AND DISCUSSION

The syntheses commenced from L-proline or D-proline by reduction with $LiAlH_4$ in THF according to the procedure of Pericas[5] to provide the corresponding prolinols (4*S*)-**3** and (4*R*)-**3** (Scheme 1). Protection of the pyrrolidine nitrogen atom as the *tert*-butyl carbamate followed by tosylation of the primary hydroxy group and cyano group displacement gave rise to nitriles (4*S*)-**4** and (4*R*)-**4** in 60% and 75% yields,

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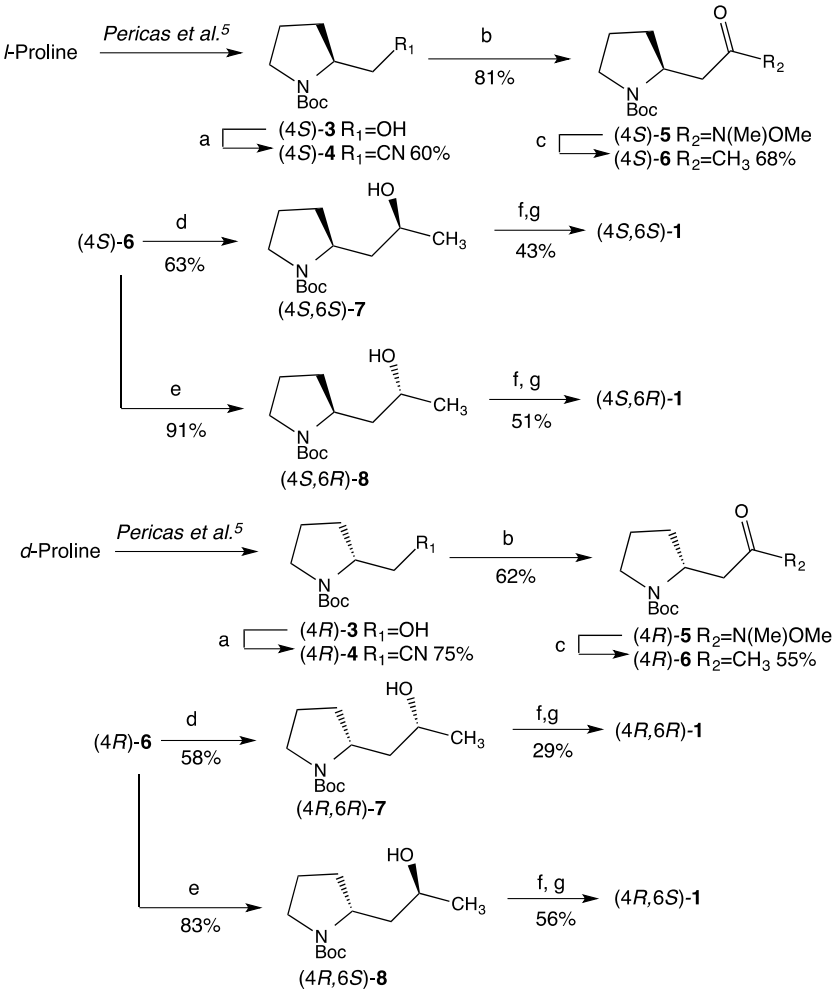
Scheme 1. Synthesis of the Four Diastereomers of Conioidine A



respectively.[6] Hydrolysis of the cyano groups under basic conditions provided the intermediate carboxylic acids, which were immediately converted to the corresponding Weinreb amides (4*R*)-**5** and (4*S*)-**5** in 81% and 62% yields, respectively. Treatment of amides **5** with CH_3MgBr in THF rapidly afforded methyl ketones (4*S*)-**6** (68%) and (4*R*)-**6** (55%). Davies[7a] has shown that stereoselective reduction of ketones **6** may be performed either with $LiAlH(OtBu)_3$ in THF at 0 °C or with $Zn(BH_4)_2$ in THF at 0 °C. Reduction of (4*S*)-**6** and (4*R*)-**6** with $Zn(BH_4)_2$ afforded the alcohols (4*S*,6*S*)-**7** (63%) and (4*R*,6*R*)-**7** (58%) (dr = 3:1; both separable from the (4*S*,6*R*) and (4*R*,6*S*) diastereomers, respectively, by silica gel chromatography).[7b] In contrast, reduction of ketones (4*S*)-**6** and (4*R*)-**6** with $LiAlH(Ot\text{-}Bu)_3$ gave rise to alcohols (4*S*,6*R*)-**8** (91%) and (4*R*,6*S*)-**8** (83%) with >20:1 diastereoselection in each case. Cleavage of the Boc group of (4*S*,6*S*)-**7**, (4*R*,6*R*)-**7**, (4*S*,6*R*)-**8**, and (4*R*,6*S*)-**8** with 1:1 TFA/CH_2Cl_2 and reaction of the corresponding amino alcohol intermediates with decanoyl chloride in the presence of Et_3N and CH_2Cl_2 for 8 h at room temperature furnished the corresponding intermediate amides, which were immediately exposed to 3 equiv of 2,3-dimethylacryloyl chloride in the presence of Et_3N (6 equiv) and DMAP (3 equiv) in CH_2Cl_2 at room temperature overnight to afford the four conioidine A diastereomers (4*S*,6*S*)-**1**, (4*R*,6*R*)-**1**, (4*S*,6*R*)-**1**, and (4*R*,6*S*)-**1** in 43%, 29%, 51%, and 56% overall yields from **7** or **8**. On the basis of comparison of the ^1H and ^{13}C NMR and specific rotation data for all four diastereomers with the values recorded for the natural product, we conclude that natural conioidine A possesses the (4*R*,6*R*) absolute configuration (see Supporting Information).

To assess the strengths of binding of the four diastereomers of conioidine A to calf thymus (CT) DNA, the competitive ethidium displacement technique was employed to obtain C_{50} values (the concentration of ligand required to achieve a 50% decrease in the fluorescence of ethidium bromide) and apparent association constants (K_{app}).[8] Titration of CT DNA (10 μM) and ethidium bromide (10 μM) with (4*R*,6*R*)-conioidine A resulted in a negligible effect on ethidium fluorescence intensity (measured at 590 nm) over the 0.01−50 μM ligand concentration range (pH = 6.81, Tris-HCl buffer); indeed, 93% of the initial ethidium fluorescence was measured at 50 μM of (4*R*,6*R*)-**1** (Figure 2a), and extrapolation of the data gave a C_{50} value of 480 μM (estimated $K_{app} = 1.8 \times 10^5$ M^{-1} bp^{-1}). Similar results were obtained in ethidium displacement studies employing (4*S*,6*S*)-**1**, (4*R*,6*S*)-**1**, and (4*S*,6*R*)-**1** (C_{50} = 470, 340, and 380 μM, respectively). Under otherwise identical experimental conditions, the positive control netropsin produced a significant decrease in ethidium emission fluorescence intensity with a directly measurable C_{50} value of 10 ± 2.5 μM ($K_{app} = (5.1 \pm 0.3) \times 10^6$ M^{-1} bp^{-1}; see Figure 2b); in contrast, the negative control dextrose showed

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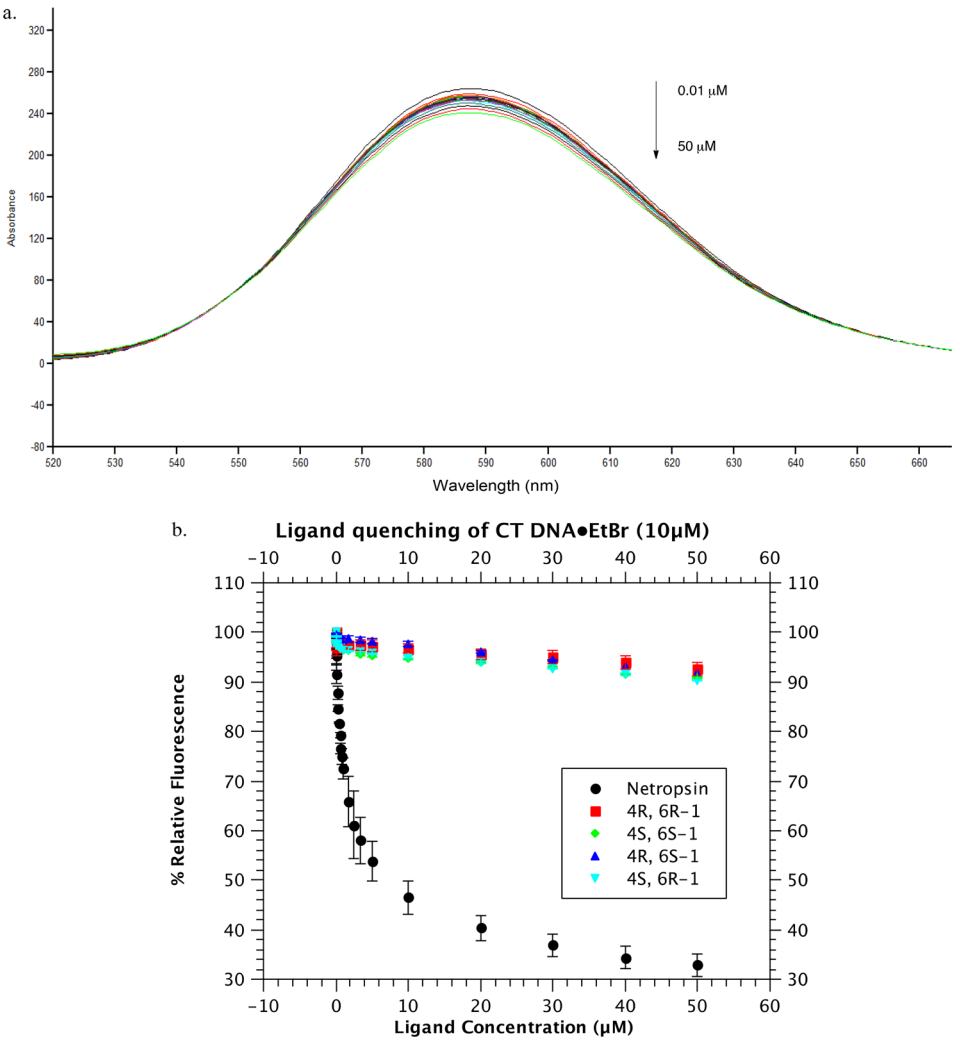

Figure 2. (a) Displacement of ethidium bromide from CT DNA by (4*R*,6*R*)-**1**. (b) Plot of % relative fluorescence vs ligand concentration for (4*R*,6*R*)-**1**, (4*S*,6*S*)-**1**, (4*R*,6*S*)-**1**, (4*S*,6*R*)-**1**, and control compound netropsin binding to CT DNA with displacement of ethidium bromide.

minimal displacement of ethidium from CT DNA with an extrapolated C_{50} value of 390 μM. Furthermore, thermal denaturation studies[9] of CT DNA employing UV spectroscopy showed minimal differences (ΔT_M = ±0.7 °C; see Supporting Information) in the helix melting temperature in the presence or absence of the four diastereomers of **1**, whereas the same experiment performed with netropsin showed strong helix stabilization (ΔT_M = +10.3 °C). These results clearly indicate that the conioidines interact relatively weakly with duplex CT DNA.

In light of these data, we explored the possibility that this family of natural products may form complexes with proteins. Numerous hydrophobic organic molecules are known to associate with the serum albumins, and binding to these proteins typically influences the apparent solubility, distribution, metabolism, and efficacy of a wide range of drugs.[10,19] Titration of the abundant plasma protein human serum albumin[11] (HSA; N form at pH 6.81, Tris-HCl buffer; 10 μM) with (4*R*,6*R*)-**1** over the 0.5−10 μM[12] range showed a significant quenching of the Trp 214 fluorescence emission at 338 nm (λ_{ex} = 280 nm; K_{SV} = (7.2 ± 0.4) × 10^4 M^{-1}, Figure 3a,b).[13,14] Analogous experiments with the (4*S*,6*S*)-**1**, (4*R*,6*S*)-**1**, and (4*S*,6*R*)-**1** stereoisomers revealed that compound (4*R*,6*S*)-**1** bound tightest to HSA (K_{SV} = (1.3 ± 0.1) × 10^5 M^{-1}), while both (4*S*,6*S*)-**1** and (4*S*,6*R*)-**1** had similar HSA binding affinities to (4*R*,6*R*)-**1** and the positive control compound virstatin (K_{SV} = (3.0 ± 0.8) × 10^4 M^{-1}; see Supporting Information and Table 1).

ITC titration[15] of HSA (57 μM) with (4*R*,6*R*)-**1** (100 μM solution, 25 °C, pH = 6.81, Tris-HCl buffer, Figure 3c) gave a binding constant K_b of (6.4 ± 0.2) × 10^4 M^{-1}, along with an enthalpy of binding, ΔH, of −1.9 ± 0.3 kcal/mol and an entropy of binding, ΔS, of 15.5 ± 0.7 cal/mol·K. ITC titration of HSA with (4*R*,6*S*)-**1** gave a binding constant K_b of (1.6 ± 0.3) × 10^5 M^{-1}, along with an enthalpy of binding, ΔH, of −1.6 ± 0.1 kcal/mol and an entropy of binding, ΔS, of 18.2 ± 0.8 cal/mol·K. The close agreement of K_{SV} and K_b values obtained from fluorescence and ITC experiments, respectively, again confirms that compound (4*R*,6*S*)-**1** is the tightest binder of HSA. It should be noted that for all diastereomers except (4*S*,6*R*)-**1** the magnitude of the entropy of binding ($-T\Delta S$ term) exceeds the magnitude of the enthalpy of binding (ΔH), suggesting that the hydrophobic effect may play an important role in the association of these compounds with HSA.[26] Competition binding experiments employing warfarin (site I marker, K_b = 2.8 × 10^5 M^{-1})[21] or ibuprofen (site II marker, K_b = 2.9 × 10^6 M^{-1})[22] and the diastereomers of **1** are currently underway to clarify which binding site (Sudlow site I or

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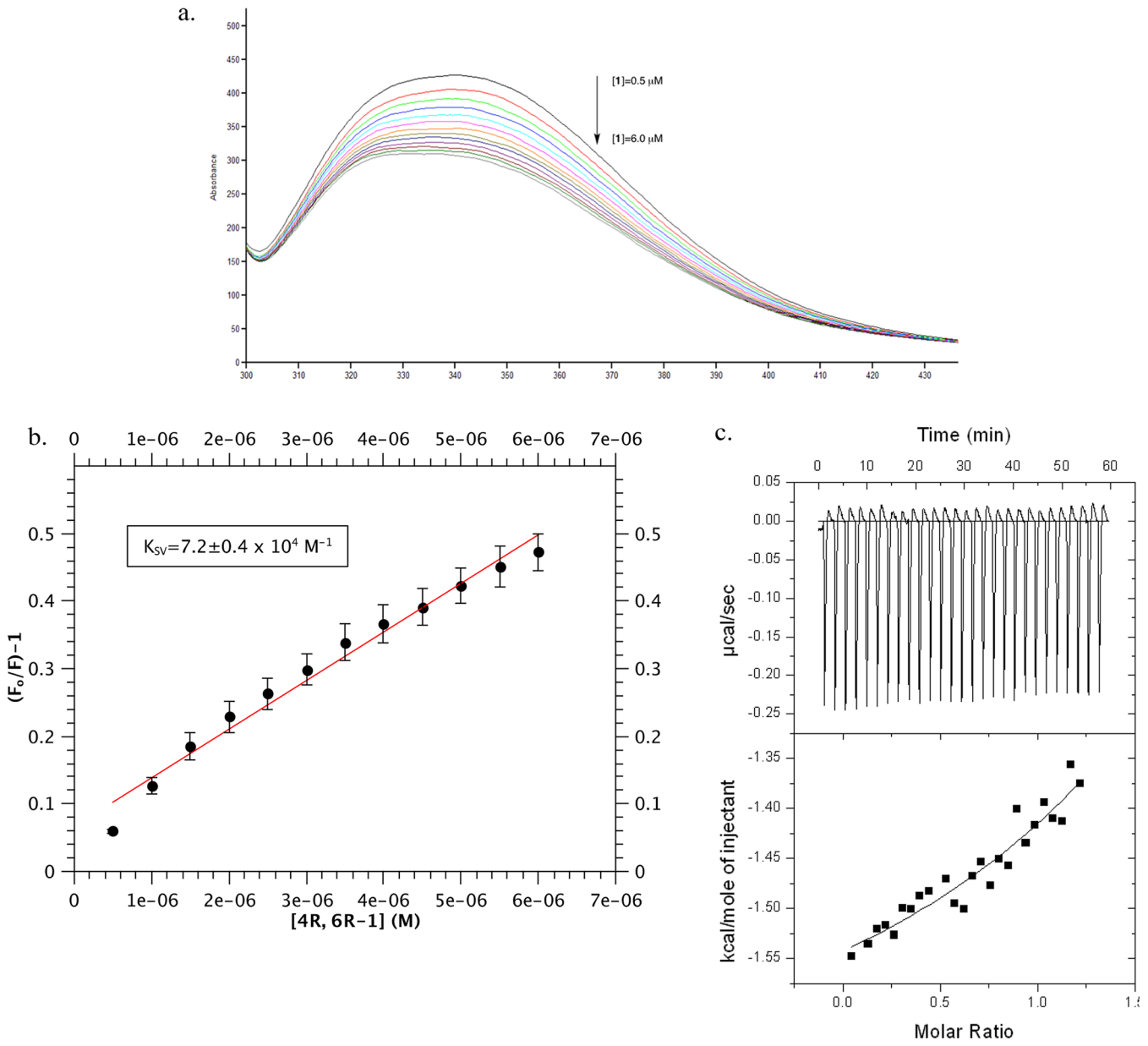

Figure 3. (a) Fluorescence quenching at 339 nm by addition of (4*R*,6*R*)-**1** to HSA. (b) Stern−Volmer plot for data represented in (a). (c) ITC trace for the binding of (4*R*,6*R*)-**1** to HSA.

Table 1. Experimental HSA Binding Data for the Four Diastereomers of 1[a]

1	K_b ($\times 10^4$ M^{-1})	ΔH (kcal/mol)	ΔS (cal/mol·K)
4*S*,6*R*	5.9 ± 1.2	−29.3 ± 0.1	−76.5 ± 0.7
4*R*,6*S*	16.0 ± 3.0	−1.6 ± 0.1	18.2 ± 0.8
4*S*,6*S*	7.2 ± 1.4	−1.8 ± 0.4	16.4 ± 1.3
4*R*,6*R*	6.4 ± 0.2	−1.9 ± 0.3	15.5 ± 0.7

[a]Binding data obtained by isothermal titration calorimetry.[15]

Sudlow site 2) on the protein is preferred by the conioidines.[16] Molecular docking studies[17] between HSA (PDB 1AO6)[18] and (4*R*,6*R*)-**1** indicate that in the lowest energy binding mode the ligand is bound to Sudlow site I in close proximity to the Trp 214 residue (see Supporting Information).

Finally, electronic circular dichrosim (ECD) spectra of HSA recorded in the 200−260 nm region in the presence of increasing concentrations of (4*R*,6*S*)-**1** revealed a marginal decrease in ellipticity of the negative Cotton effects at 208 and 220 nm characteristic of the α-helical structure of the protein (Figure 4). These results are consistent with a minor structural perturbation of HSA upon ligand binding, resulting in a slight decrease in α-helical content. Highly similar results have been reported for the binding of caffeine[19] and scutellarin[20] to HSA.

In summary, the relative and absolute configurations of conioidine A [(4*R*,6*R*)-**1**] have been determined through chemical synthesis of the four possible diastereomers and comparison of their spectroscopic data with those reported for the natural product. In comparison to the known minor groove binding agent netropsin, the four diastereomers of conioidine A are relatively weak DNA-binding compounds; in contrast, the compounds show good affinity for HSA, with the (4*R*,6*S*) diastereomer having the highest measured binding constant ($(1.6 \pm 0.3) \times 10^5$ M^{-1}). Preliminary cell viability investigations utilizing MTT assays[23] suggest that (4*R*,6*R*)-**1** is cytotoxic toward MCF-7 cells and that the presence of

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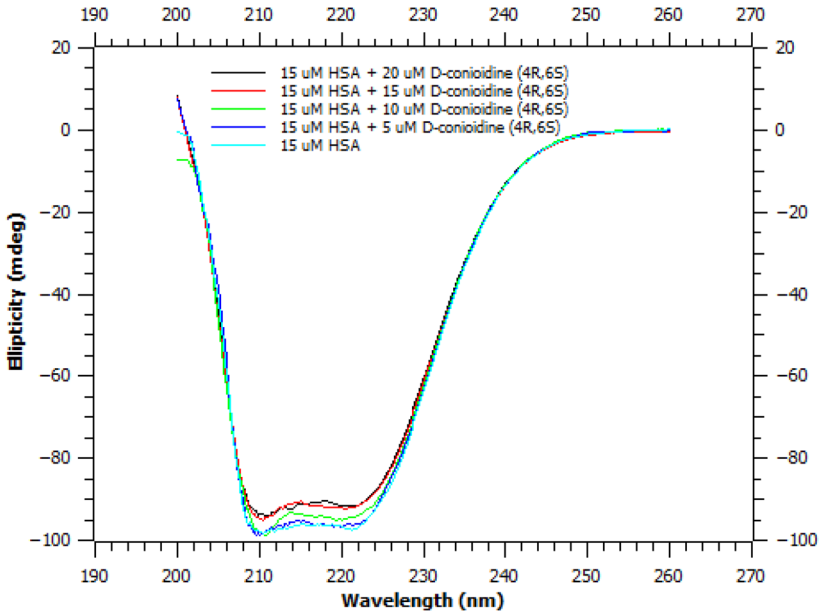

Figure 4. The 200−260 nm region of the ECD spectra of solutions of HSA (15 μM) in the absence (light blue line) and presence of various concentrations of (4*R*,6*S*)-**1**: blue line 5.0 μM (4*R*,6*S*)-**1**; green line 10 μM (4*R*,6*S*)-**1**; red line 15 μM (4*R*,6*S*)-**1**; black line 20 μM (4*R*,6*S*)-**1**.

exogenous CT DNA attenuates cell killing, in line with the observations of Chan.[1] Full details on these studies will be reported elsewhere in due course.

EXPERIMENTAL SECTION

General Experimental Procedures. All reagents and solvents were purchased and used without further purification. Distilled water was used in all of the experiments. Organic extracts were dried over Na_2SO_4, filtered, and concentrated using a rotary evaporator at aspirator pressure (20−30 mm Hg). Chromatography refers to flash chromatography and was carried out on SiO_2 (silica gel 60, 230−400 mesh). All glassware used in the reactions described below were flame-dried under vacuum and then flushed with argon gas at room temperature prior to the addition of reagents and solvents. ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ at 400 and 100 MHz, respectively, using Me_4Si as internal standard. Chemical shifts are reported in ppm downfield (δ) from Me_4Si. UV spectra were recorded on a diode array spectrophotometer over the 190−500 nm range with subtraction of a solvent blank. Fluorescence spectra were recorded over the 520−670 nm range for ethidium bromide displacement experiments (the maximum emission wavelength was 590 nm when the excitation wavelength was 520 nm; ex slit (nm) = 10.0; em slit (nm) = 10.0; scan speed (nm/min) = 200) and over the 320−410 nm range for HSA binding experiments (the maximum emission wavelength was 338 nm when the excitation wavelength was 280 nm; ex slit (nm) = 10.0; em slit (nm) = 10.0; scan speed (nm/min) = 200). ITC thermograms were recorded for titrations performed at 25 °C under the following conditions: DP = 6, 307 rpm, 5 μL injections, 10 s duration with 130 s, 2 s filter, initial delay of 60 s, total of 45 injections.

*(2S)-tert-Butyl 2-(2-Oxopropyl)pyrrolidine-1-carboxylate [(4S)-**6**] and (2R)-tert-Butyl 2-(2-Oxopropyl)pyrrolidine-1-carboxylate [(4R)-**6**].* Nitrile (4*S*)-**4**[24] or (4*R*)-**4**[5] (1.0 g, 4.7 mmol) was dissolved in MeOH (4.7 mL) and 3 N NaOH (4.7 mL, 14.1 mmol) and heated to 100 °C for one hour, at which time a homogeneous solution was obtained. The solution was cooled to room temperature and concentrated *in vacuo* to remove MeOH. Et_2O (10 mL) was added, and the solution was acidified with 3 N HCl (4.5 mL). The phases were separated, and the aqueous layer was extracted with Et_2O (2 × 25 mL). The combined organic extracts were once washed with 20 mL of saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude acid (4.7 mmol) was dissolved in CH_2Cl_2 (11 mL) and cooled to 0 °C. Carbonyl diimidazole (856 mg, 5.28 mmol) was added, and the solution was allowed to stir at room temperature for 30 min. The solution was cooled to 0 °C, Et_3N (1.68 mL, 12 mmol) and *N*-methyl-*N*-methoxyamine hydrochloride (582 mg, 6 mmol) were added, and the solution was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2 (10 mL) and saturated $NaHCO_3$ solution (20 mL), and the phases were separated. The aqueous layer was extracted with Et_2O (2 × 25 mL). The combined organic extract was washed once with 20 mL of saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude Weinreb amide (~4 mmol) was dissolved in THF (10 mL) and cooled to 0 °C. A solution of CH_3MgBr (1.5 mL, 4.5 mmol, 3 M in Et_2O) was added dropwise, and the solution was allowed to stir at room temperature for 4 h. The reaction mixture was quenched with saturated NH_4Cl solution (10 mL), and Et_2O (10 mL) was added. The phases were separated, and the aqueous layer was extracted with Et_2O (2 × 25 mL). The combined organic extract was washed once with 20 mL of saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Purification of the residue by flash chromatography (SiO_2, 20:1 → 4:1 hexanes/EtOAc) afforded ketone (4*S*)-**6** (586.8 mg, 2.6 mmol, 55%) and ketone (4*R*)-**6** (363 mg, 1.6 mmol, 34%). (4*S*)-**6** (as a mixture of carbamate rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.16 (m, 1H), 3.32 (m, 2H), 3.15, 2.95 (dd, J = 15.3, 15.6 Hz, 1H), 2.44 (m, 1H), 2.17 (s, 3H), 2.08 (m, 1H), 1.84 (m, 2H), 1.67 (m, 1H), 1.47 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.5, 154.3, 79.5, 53.4, 47.8, 46.4, 31.5, 30.5, 28.4, 23.5; HRMS (ESI) calculated for $C_{12}H_{21}NNaO_3$ 250.1419, found 250.1376 $(M + Na)^+$; $[\alpha]^{25}_D$ = −36.5 (*c* 0.02, CH_2Cl_2). These data are in full accord with those reported for (4*S*)-**6**.[25] (4*R*)-**6** (as a mixture of carbamate rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.09 (m, 1H), 3.29 (t, J = 5.9 Hz, 2H), 2.96 (d, J = 16.0 Hz, 1H), 2.37 (dd, J = 9.6, 16.0 Hz, 1H), 2.11 (s, 3H), 2.01 (m, 1H), 1.79 (m, 2H), 1.60 (m, 1H), 1.42 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.3, 154.2, 79.3, 53.4, 48.2, 46.3, 31.1, 30.4, 28.5, 23.2; HRMS (ESI) calculated for $C_{12}H_{21}NNaO_3$ 250.1419, found 250.1400 $(M + Na)^+$; $[\alpha]^{25}_D$ = +18.6 (*c* 0.05, $CHCl_3$). These data are in full accord with those reported for (4*R*)-**6**.[7]

*(2S)-tert-Butyl 2-[(2S)-2-Hydroxypropyl]Pyrrolidine-1-carboxylate [(4S,6S)-**7**] and (2R)-tert-Butyl 2-[(2R)-2-Hydroxypropyl]-Pyrrolidine-1-carboxylate [(4R,6R)-**7**].* Ketone (4*S*)-**6** or (4*R*)-**6** (120 mg, 0.53 mmol) was dissolved in THF (1 mL), and the mixture was cooled to 0 °C; $Zn(BH_4)_2$ (1 mL of a 4 M solution, 4 mmol) was

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J. Nat. Prod. XXXX, XXX, XXX−XXX

added, and the mixture was stirred at 0 °C for 6 h. The mixture was quenched by addition of a saturated solution of Rochelle's salt (5 mL) and stirred at room temperature for 2 h. After dilution of the mixture with EtOAc (10 mL), the phases were separated and the aqueous layer was extracted with Et_2O (2 × 25 mL). The combined organic extract was washed once with 20 mL of saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Purification of the residue by flash chromatography (SiO_2, 10:1 → 3:1 hexanes/EtOAc) afforded alcohol (4*S*,6*S*)-**7** (76.4 mg, 0.33 mmol, 63%) or (4*R*,6*R*)-**7** (70.3 mg, 0.31 mmol, 58%). (4*S*,6*S*)-**7** (as a mixture of carbamate rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.13 (br s, 1H), 4.15 (m, 1H), 3.71 (m, 1H), 3.29 (t, J = 5.8 Hz, 2H), 1.96−1.82 (m, 3H), 1.58 (m, 1H), 1.44 (s, 9H), 1.17 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.5, 79.8, 63.8, 53.7, 46.5, 45.5, 31.1, 28.4, 23.5, 22.5; HRMS (ESI) calculated for $C_{12}H_{23}NNaO_3$ 252.1576, found 252.1555 $(M + Na)^+$; $[\alpha]^{25}_D$ = −5.8 (*c* 0.01, $CHCl_3$). These data are in full accord with those reported for (4*S*,6*S*)-**7**.[7] (4*R*,6*R*)-**7** (as a mixture of carbamate rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) 4.16 (m, 1H), 3.72 (m, 1H), 3.30 (m, 2H), 1.99−1.85 (m, 3H), 1.60 (m, 1H), 1.47 (s, 9H), 1.40 (m, 2H), 1.18 (d, J = 6.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.5, 79.8, 63.7, 53.8, 46.4, 45.6, 31.1, 28.4, 23.4, 22.6; HRMS (ESI) calculated for $C_{12}H_{23}NNaO_3$ 252.1576, found 252.1478 $(M + Na)^+$; $[\alpha]^{25}_D$ = +7.5 (*c* 0.02, $CHCl_3$). These data are in full accord with those reported for (4*R*,6*R*)-**7**.[7]

*(2S)-tert-Butyl 2-[(2R)-2-Hydroxypropyl]pyrrolidine-1-carboxylate [(4S,6R)-**8**] and (2R)-tert-Butyl 2-[(2S)-2-Hydroxypropyl]-pyrrolidine-1-carboxylate [(4R,6S)-**8**].* Ketone (4*S*)-**6** or (4*R*)-**6** (320 mg, 1.41 mmol) was dissolved in THF (5 mL), and the mixture was cooled to −78 °C; $LiAlH(OtBu)_3$ (1.07 g, 4.24 mmol, 3 equiv) was added, and the mixture was warmed to 0 °C and stirred at this temperature for 6 h. The mixture was quenched with a few drops of water and diluted with EtOAc (10 mL). The solution was cooled to 0 °C, filtered through Celite, and concentrated *in vacuo*. Purification of the residue by flash chromatography (SiO_2, 10:1 → 3:1 hexanes/EtOAc) afforded alcohol (4*S*,6*R*)-**8** (293 mg, 1.28 mmol, 91%) or (4*R*,6*S*)-**8** (267 mg, 1.17 mmol, 83%). (4*S*,6*R*)-**8** (as a mixture of carbamate rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) 3.95 (br s, 1H), 3.80 (m, 1H), 3.30 (m, 2H), 1.97 (m, 1H), 1.81 (m, 2H), 1.62 (m, 1H), 1.42 (s, 9H), 1.16 (d, J = 5.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 155.4, 79.5, 66.3, 55.5, 46.3, 45.3, 32.1, 28.4, 23.9, 23.7; HRMS (ESI) calculated for $C_{12}H_{23}NNaO_3$ 252.1576, found 252.1597 $(M+Na)^+$; $[\alpha]^{25}_D$ = −24.8 (*c* 0.08, $CHCl_3$). These data are in full accord with those reported for (4*S*,6*R*)-**8**.[7] (4*R*,6*S*)-**8** (as a mixture of carbamate rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) 3.89 (br s, 1H), 3.65 (m, J = 2.9 Hz, 1H), 3.62 (br s, 1H), 3.24 (m, 2H), 1.91−1.86 (m, 1H), 1.74 (m, 3H), 1.59 (m, 1H), 1.37 (s, 9H), 1.12 (d, J = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.2, 79.4, 66.0, 55.2, 46.2, 44.9, 31.7, 28.4, 23.9, 23.7; HRMS (ESI) calculated for $C_{12}H_{23}NNaO_3$ 252.1576, found 252.1579 $(M + Na)^+$; $[\alpha]^{25}_D$ = +33.3 (*c* 0.02, $CHCl_3$). These data are in full accord with those reported for (4*R*,6*S*)-**8**.[7]

(4S,6S)-Conioidine A, (4R,6R)-Conioidine A, (4R,6S)-Conioidine A, and (4S,6R)-Conioidine A. Alcohol (4*S*,6*S*)-**7**, (4*R*,6*R*)-**7**, (4*S*,6*R*)-**8**, or (4*R*,6*S*)-**8** (150 mg, 0.66 mmol) was dissolved in 1:1 TFA/CH_2Cl_2 (1.5 mL) at room temperature, and the mixture was stirred for 30 min, at which time it was concentrated *in vacuo*. The crude residue was azeotroped with benzene (5 mL) twice, and CH_2Cl_2 (1.5 mL) was added. Et_3N (0.25 mL, 1.6 mmol) was added, and the mixture was cooled to 0 °C. Decanoyl chloride (0.15 mL, 0.72 mmol) was added dropwise, and the mixture was warmed to room temperature and stirred overnight. A solution of saturated $NaHCO_3$ (5 mL) was added, and the layers were separated. The aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic extract was washed once with 20 mL of saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude intermediate alcohol (~189 mg, 0.67 mmol) was dissolved in CH_2Cl_2 (2 mL) and cooled to 0 °C. Pyridine (0.216 mL, 2.68 mmol), DMAP (163 mg, 1.34 mmol), and 2,3-dimethylacryloyl chloride (158 mg, 1.34 mmol) were added successively, and the mixture was allowed to warm to room temperature and stir overnight. CH_2Cl_2 (10 mL) and a saturated solution of $NaHCO_3$ were added (10 mL), and the layers were separated. The aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic extract was washed once with 20 mL of saturated aqueous NaCl, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. Purification of the residue by flash chromatography (SiO_2, 5:1 hexanes/EtOAc → 2:1 hexanes/EtOAc) afforded the four diastereomers (4*S*,6*S*)-**1**, (4*R*,6*R*)-**1**, (4*S*,6*R*)-**1**, and (4*R*,6*S*)-**1**. (4*S*,6*R*)-**1** (101 mg, 0.344 mmol, 51%, as a mixture of amide rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.89 (m, 1H), 4.99 (m, 1H), 4.20, 3.85 (m, 1H), 3.47−3.40 (m, 2H), 2.31 (t, J = 7.6 Hz, 1H), 2.21 (t, J = 8.1 Hz, 1H), 1.89−1.75 (m, 5H), 1.77 (s, 3H), 1.74 (d, J = 7.0 Hz, 3H), 1.64 (m, 2H), 1.44 (m, 1H), 1.27 (d, J = 6.2 Hz, 3H), 1.27−1.24 (m, 12H), 0.86 (t, J = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 167.8, 137.4, 137.1, 128.8, 128.6, 68.5, 68.3, 54.4, 53.8, 46.7, 45.3, 41.8, 39.2, 35.1, 34.3, 31.8, 30.1, 29.6, 29.5, 29.4, 29.3, 29.2, 25.5, 24.8, 23.9, 22.6, 21.8, 20.9, 20.5, 14.4, 14.0, 12.1, 12.0; HRMS (ESI) calculated for $C_{22}H_{40}NO_3$ 366.2990, found 366.2958 $(M + H)^+$; $[\alpha]^{25}_D$ = −9.9 (*c* 0.01, MeOH). (4*S*,6*S*)-**1** (84 mg, 0.28 mmol, 43%, as a mixture of amide rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (m, 1H), 4.96 (q, J = 6.4 Hz, 1H), 4.08, 3.83 (m, 1H), 3.38 (m, 2H), 2.21 (t, J = 8.3 Hz, 2H), 2.08 (m, 1H), 1.91−1.78 (m, 9H), 1.66−1.58 (m, 3H), 1.31−1.25 (m, 16H), 0.88 (t, J = 6.7 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 167.5, 136.7, 129.0, 69.6, 54.9, 46.6, 39.3, 35.0, 31.8, 29.5, 29.4, 29.2, 24.7, 24.1, 22.6, 20.0, 14.2, 14.0, 11.9; HRMS (ESI) calculated for $C_{22}H_{39}NNaO_3$ 388.2828, found 388.2838 $(M + Na)^+$; $[\alpha]^{25}_D$ = −38.5 (*c* 0.01, MeOH). (4*R*,6*S*)-**1** (110 mg, 0.37 mmol, 56%, as a mixture of amide rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.90 (m, 1H), 5.00 (m, 1H), 4.20, 3.85 (m, 1H), 3.47−3.38 (m, 2H), 2.28 (t, J = 7.8 Hz, 1H), 2.25 (t, J = 7.1 Hz, 2H), 1.91−1.76 (m, 9H), 1.64 (m, 2H), 1.44 (m, 1H), 1.41−1.27 (m, 16H), 0.89 (t, J = 6.6 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 167.8, 137.3, 128.8, 68.5, 54.4, 53.8, 46.7, 45.3, 41.8, 39.2, 35.1, 34.3, 31.8, 30.1, 29.6, 29.5, 29.4, 29.3, 25.5, 24.8, 23.9, 22.6, 21.8, 20.9, 20.5, 14.4, 14.0, 12.1; HRMS (ESI) calculated for $C_{22}H_{40}NO_3$ 366.2990, found 366.3042 $(M + H)^+$; $[\alpha]^{25}_D$ = +8.0 (*c* 0.01, MeOH). (4*R*,6*R*)-**1** (70 mg, 0.19 mmol, 29%, as a mixture of amide rotamers): ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.82 (q, J = 4.3 Hz, 1H), 4.96 (q, J = 4.0 Hz, 1H), 4.08 (m, 1H), 3.41−3.35 (m, 2H), 2.21 (t, J = 8.1 Hz, 2H), 2.08 (m, 1H), 1.91−1.80 (m, 6H), 1.78 (d, J = 8.0 Hz, 3H), 1.64 (m, 3H), 1.32−1.30 (m, 13H), 1.31 (d, J = 8.0 Hz, 3H), 0.89 (t, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 167.5, 136.7, 129.0, 69.6, 54.9, 46.6, 39.3, 35.1, 31.8, 29.5, 29.2, 24.7, 24.1, 22.6, 20.0, 14.3, 14.0, 12.0; HRMS (ESI) calculated for $C_{22}H_{39}NaO_3$ 388.2828, found 388.2832 $(M + Na)^+$; $[\alpha]^{25}_D$ = +25.7 (*c* 0.03, MeOH). These data are in accord with those reported for conioidine A.[1]

Competitive Ethidium Bromide Displacement Experiments. Constant concentrations of CT-DNA (10 μM) and EtBr (10 μM) (each in Tris-HCl buffer, pH 6.81) were titrated with increasing concentrations of the diastereomers of **1** (from 1 mM and 100 μM stock solutions in Tris-HCl buffer, pH 6.81) or netropsin. The maximum emission wavelength was 490 nm when the excitation wavelength was 520 nm. Fluorescence titrations were recorded from 520 to 692 nm after an equilibration period of 3 min: ex slit (nm) = 10.0; em slit (nm) = 10.0; scan speed (nm/min) = 200.

Thermal Denaturation Studies. UV thermal denaturation samples (2 mL) were prepared by mixing CT-DNA in Tris-HCl buffer (pH 6.81) in 1 cm path length quartz cuvettes. The DNA to ligand ratio was 40:1. Absorbance readings were taken at 260 nm for temperatures ranging from 25 to 95 °C. Temperature was increased gradually with a speed of 1 °C/min with an absorbance reading every 2 °C. First derivative plots were used to determine the T_M value.

ECD Studies. Small aliquots (0.6−5.0 μL) of a concentrated (4*R*,6*S*)-**1** solution (1 mM) were added to a solution of HSA (15 μM in pH = 6.81 in Tris-HCl buffer), inverted twice, and incubated for 5 min at 20 °C. The ECD spectra were recorded as an average of three scans from 220 to 310 nm, and data recorded in 0.1 nm increments.

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J. Nat. Prod. XXXX, XXX, XXX−XXX

ITC Studies. Ligands **1** (100 μM in pH = 6.81 Tris-HCl buffer) were titrated against HSA (57 μM in pH = 6.81, Tris-HCl buffer) at 25 °C. ITC thermograms were recorded for titrations under the following conditions: DP = 6, 307 rpm, 5 μL injections, 10 s duration with 130 s, 2 s filter, initial delay of 60 s, total of 45 injections.

Molecular Docking Studies. Compound (4*R*,6*R*)-**1** was minimized using Spartan'14 for Macintosh. Molecular docking studies were performed with (4*R*,6*R*)-**1** and HSA (PDB 1AO6)[18] using AutoDock Vina.[17] The search space included both Sudlow sites I and II.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00871.

Experimental details, characterization data, and ^{1}H and ^{13}C NMR spectra for all compounds in Scheme 1; fluorescence, UV, ECD, and ITC binding data for the four diastereomers of **1** (PDF)

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Notes

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CH_2Cl_2 for 48 h at 37 °C. No reaction was observed. Furthermore, we exposed (4*S*,6*S*)-**1** to ethanethiol (5 molar equiv) and a catalytic quantity (10 mol %) of the base DBU in CH_2Cl_2 for 48 h at 37 °C. Again, minimal conversion took place. These observations indicate that the tigloate functional group of the conioidines is not highly reactive toward conjugate addition of thiols or their conjugate bases. (b) Since all of the cysteine side chains of HSA are engaged in disulfide linkages, we anticipate that major structural changes to the protein would be required for covalent attachment via cysteine thiol conjugate addition to the enoate side chain of the natural product. However, the changes observed during ECD titration of HSA with (4*R*,6*S*)-**1** (Figure 4) indicate only a minor structural perturbation of the protein upon ligand binding, which is consistent with what is observed for the noncovalent binding of caffeine to HSA: Wu, Q.; Li, C.-H.; Hu, Y.-J.; Liu, Y. *Sci. China, Ser. B: Chem.* **2009**, *52*, 2205. (c) HSA is a carrier protein in the blood that is known to bind numerous small hydrophobic organic molecules in a noncovalent fashion, which is attested to by numerous X-ray crystal structures of ligand−HSA complexes; see: Zhu, L.; Yang, F.; Chen, L.; Meehan, E. J.; Huang, M. *J. Struct. Biol.* **2008**, *162*, 40. Yamaguchi, S.; Aldini, G.; Ito, S.; Morishita, N.; Shibata, T.; Vistoli, G.; Carini, M.; Uchida, K. *J. Am. Chem. Soc.* **2010**, *132*, 824. Guo, S.; Shi, X.; Yang, F.; Chen, L.; Meehan, E. J.; Bian, C.; Huang, M. *Biochem. J.* **2009**, *423*, 23. and refs 16c and 16d.

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J. Nat. Prod. XXXX, XXX, XXX−XXX