Acta Crystallographica Section C
Crystal Structure Communications
ISSN 0108-2701

cyclo(-Cha–Oxz–D-Val–Thz–Ile–Oxz–D-Val–Thz-) *N*,*N*-dimethylacetamide dihydrate: a square form of cyclohexylalanine-incorporated ascidiacyclamide having the strongest cytotoxicity

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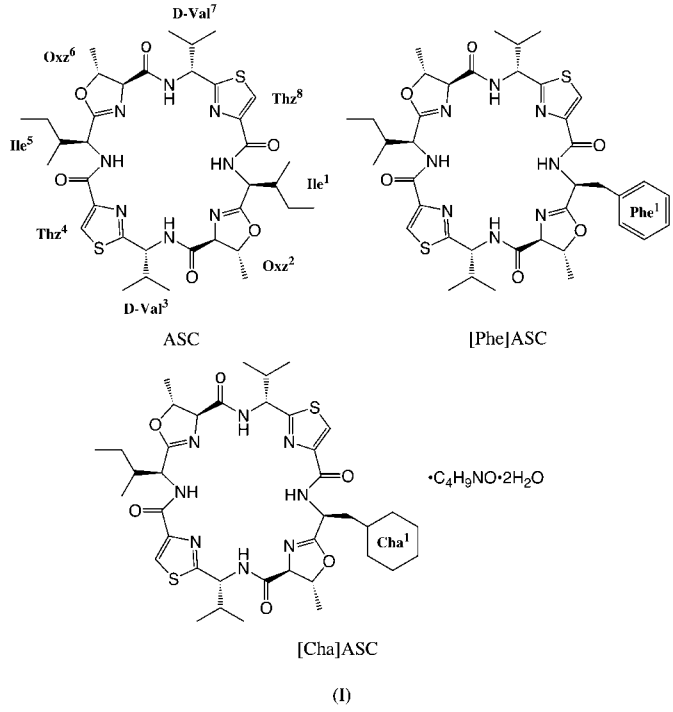
Received 9 June 2003
Accepted 30 June 2003
Online 9 August 2003

The title compound, 1-cyclohexylmethyl-1-de(1-methylpropyl)ascidiacyclamide *N*,*N*-dimethylacetamide dihydrate, $C_{39}H_{56}N_8O_6S_2{\cdot}C_4H_9NO{\cdot}2H_2O$, a cyclohexylalanine-incorporated ascidiacyclamide analogue ([Cha]ASC), shows a square form similar to natural ASC. On the other hand, CD (circular dichroism) spectra showed [Cha]ASC to have a folded structure in solution, making it the second known analogue to show a discrepancy between its crystal and solution structures. Moreover, the cytotoxicity of [Cha]ASC (ED_{50} = 5.6 μg ml^{-1}) was approximately two times stronger than that of natural ASC or a related phenylalanine-incorporated analogue, *viz*. cyclo(-Phe–Oxz–D-Val–Thz–Ile–Oxz–D-Val–Thz-) ([Phe]ASC), and was confirmed to be associated with the square form. However, [Phe]ASC was previously shown to be folded in the crystal structure, which suggests that the difference between the aromatic and aliphatic rings affects the molecular folding of the ASC molecule.

Comment

Ascidiacyclamide (ASC), cyclo(-L-Ile–L-Oxz–D-Val–Thz-)$_2$, is a cytotoxic cyclic peptide isolated from tunicate that contains the unusual amino acids oxazoline (Oxz) and thiazole (Thz) (Hamamoto *et al.*, 1983). X-ray diffraction analyses have revealed ASC and its analogues to have either square or folded structures (Ishida *et al.*, 1987, 1988, 1992; Schmitz *et al.*, 1989; In *et al.*, 1993). In this regard, our findings suggest that substitution of an amino acid that disturbs the C_2-symmetry can affect the structure of the resultant ASC analogue, *viz*. the folding of the peptide is seen to be related to the bulkiness of the incorporated amino acid (Asano, Doi *et al.*, 2001; Doi *et al.*, 2001; Asano, Yamada *et al.*, 2002). On the other hand, incorporation of an amino acid of appropriate size results in a square structure and associated strong cytotoxicity (Asano, Minoura *et al.*, 2002). One exception is the phenylalanine-incorporated analogue, *viz*. cyclo(-Phe–Oxz–D-Val–Thz–Ile–Oxz–D-Val–Thz-) ([Phe]ASC), in which cytotoxicity is conserved in the folded configuration (Doi *et al.*, 1999). This is notable, as [Phe]ASC contains one of the bulkiest amino acids and is the only analogue containing an aromatic amino acid. Therefore, to better understand the determinants of the



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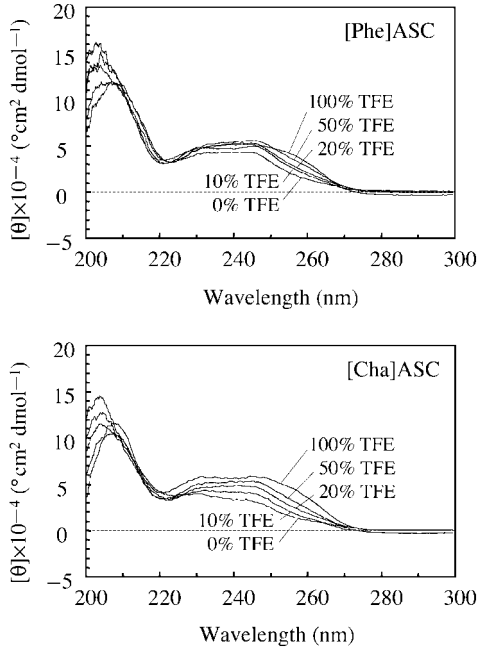

Figure 1
CD spectra for [Phe]ASC and [Cha]ASC. TFE was added to MeCN solutions at 0, 10, 20, 50 and 100% concentrations.

  DOI: 10.1107/S0108270103014495

structure and cytotoxicity of ASC analogues, we incorporated a cyclohexylalanine, which has an aliphatic six-membered ring, into ASC.

The CD (circular dichroism) spectra of [Phe]ASC and [Cha]ASC, which were measured by titration using two solvents, acetonitrile (MeCN) and 2,2,2-trifluoroethanol (TFE), were found to be similar (Fig. 1). Addition of TFE provides a special environment, different from that provided by MeCN (Fioroni *et al.*, 2000). Nevertheless, addition of TFE elicited no drastic change in the spectrum. It is known that in the square form, ASC analogues show small negative Cottons at about 250 nm (Asano, Minoura *et al.*, 2002). Since positive Cottons in the range 200–300 nm can be a sign of folding, these CD spectra imply that both [Phe]ASC and [Cha]ASC are in a folded state in solution. Indeed, the crystal structure of [Phe]ASC is known to be folded (Doi *et al.*, 1999).

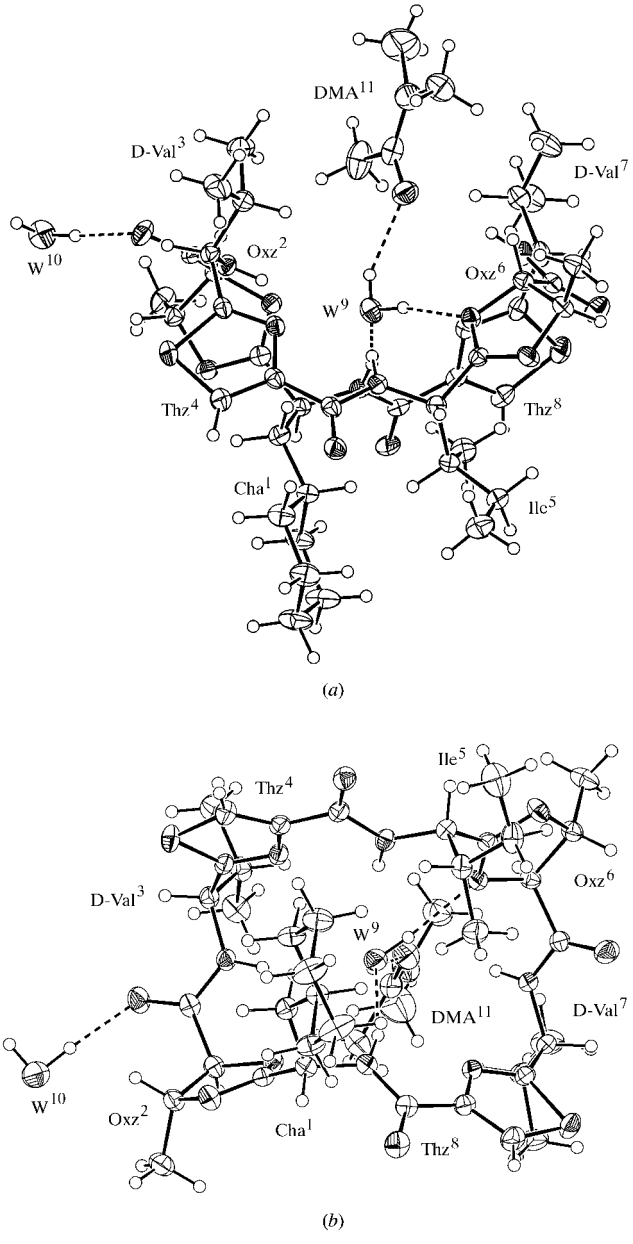

Figure 2
The molecular structure of [Cha]ASC, with displacement ellipsoids at the 50% probability level. The peptide molecule is viewed from (*a*) the side and (*b*) the top of the peptide ring.

The crystal structure of [Cha]ASC is shown in Fig. 2. There are two water molecules (W^9 and W^{10}) and one *N*,*N*-dimethylacetamide molecule (DMA^{11}) in the asymmetric unit. In contrast to the suggestion of the CD spectra, the peptide ring shows a square form similar to previously described structures (Ishida *et al.*, 1987), and the peptide and solvent molecules interact *via* hydrogen bonds (Table 1). Water molecule W^9 (O1_9) is hydrogen bonded to atoms N_1 (Cha^1) and N_6 (Oxz^6), as well as to atom O1_11 (DMA^{11}), and was finally located at a deep position within the opened peptide ring, which is reminiscent of the anchored water molecule observed in the ethanol- and water-solvated ASC crystal (In *et al.*, 1994). The other water molecule, *viz*. W^{10} (O2_10), bridges two adjacent peptide molecules (O_2 and O_4), which also interact through C—H···O hydrogen bonds (C*B*_4···O_2 and C*A*_2···O_8). This type of interaction was also observed in dimeric ASC (Asano, Taniguchi *et al.*, 2001), but this is the first observation in an ASC analogue.

The ED_{50} for the toxicity of [Cha]ASC in P388 lymphocytic leukemia cells was 5.6 μg ml^{-1}. This is the strongest toxicity yet reported for an ASC analogue (Doi *et al.*, 1999; Asano, Minoura *et al.*, 2002) and is approximately two times stronger than natural ASC (ED_{50} = 10.5 μg ml^{-1}) or [Phe]ASC (ED_{50} = 11.8 μg ml^{-1}). That the square structure of [Cha]ASC is the most cytotoxic strongly supports the association between the square form and cytotoxicity.

We did not determine the cause of the discrepancy between the structures of [Cha]ASC in the solid and solution states, though a similar discrepancy was previously observed for the Leu-incorporated analogue, *viz*. cyclo(-Leu–Oxz–D-Val–Thz–Ile–Oxz–D-Val–Thz-) ([Leu]ASC; Asano, Minoura *et al.*, 2002). The number of C atoms in Cha is the same as in Phe, making the aromaticity of the latter the only difference between [Cha]ASC and [Phe]ASC. It therefore appears likely that it is this aromaticity that accounts for the cytotoxicity of the folded structure of [Phe]ASC.

Experimental

Peptide synthesis and crystallization: [Cha]ASC was synthesized as described previously (Hamada *et al.*, 1985, 1987). ^{1}H NMR spectra of [Cha]ASC (recorded using a Varian Inova 500 at 300 K in dimethyl sulfoxide-d_6): δ_H 7.70 (1H, *br*, Ile–NH), 7.70 (1H, *br*, Cha–NH), 7.57 (2H, *s*, Thz–H), 7.32 (1H, *br*, D-Val–NH), 7.29 (1H, *br*, D-Val–NH), 5.20–5.25 (2H, *m*, D-Val–CαH), 4.90–4.97 (1H, *m,* Cha–CαH), 4.90–4.97 (2H, *m*, Oxz–CβH), 4.72–7.75 (1H, *m*, Ile–CαH), 4.36 (2H, *d*, *J* = 4.12 Hz, Oxz–CαH), 2.31–2.35 (2H, *m*, D-Val–CβH), 2.05–2.13 (1H, *m*, Ile–CβH), 1.81–1.95 (1H, *m*, Cha–CβH$_2$), 1.65–1.79, 0.87–0.95 (11H, *m*, Cha–C$_6$H$_{11}$), 1.52–1.56 (1H, *m*, Cha–CβH$_2$), 1.49 (3H, *d*, *J* = 6.41 Hz, Oxz–CγH$_3$), 1.47 (3H, *d*, *J* = 6.41 Hz, Oxz–CγH$_3$), 1.43–1.53 (1H, *m*, Ile–Cγ2H$_2$), 1.26–1.33 (1H, *m,* Ile–Cγ2H$_2$), 1.14–1.20 (12H, *m*, D-Val–CγH$_3$ × 2), 0.96 (3H, *d*, *J* = 6.86 Hz, Ile–Cγ1H$_3$), 0.84 (3H, *t,* *J* = 7.32 Hz, Ile–CγH$_3$). [Cha]ASC (7–8 mg) was dissolved in 0.2 ml of *N*,*N*-dimethylformamide (DMF) or *N*,*N*-dimethylacetamide (DMA), after which approximately 0.02 ml of water was added to each solution. Crystals grew from both solutions over a period of about a week

with different crystal forms, but the crystals obtained from DMF were not suitable for structure determination.

Circular dichroism: CD spectra were measured at 298 K using a JASCO 500A (JASCO, Tokyo, Japan) dichrograph. The peptide concentration was 0.04 m*M* and the path length was 1 cm. The spectra were scanned at a rate of 5 nm min^{-1}, with a 0.1 nm interval uptake to a computer. Data were averaged at each 1 nm and plotted.

Cytotoxicity: the cytotoxicities of ASC analogues were evaluated using P-388 lymphocytic leukemia cells, essentially as described previously, with some modification (Kohda, Ohta, Kawazoe *et al.*, 1989; Kohda, Ohta, Yokoyama *et al.*, 1989). All assays were performed three times. Semilogarithmic plots were constructed from the averaged data and the effective dose of the peptide required to inhibit cell growth by 50% (ED_{50}) was determined.

Crystal data

$C_{39}H_{56}N_8O_6S_2\cdot C_4H_9NO\cdot 2H_2O$
M_r = 920.19
Monoclinic, $P2_1$
a = 13.283 (2) Å
b = 14.153 (2) Å
c = 13.452 (2) Å
β = 105.252 (2)°
V = 2439.8 (7) $Å^3$
Z = 2
D_x = 1.253 Mg m^{-3}
Mo $K\alpha$ radiation
Cell parameters from 9020 reflections
θ = 2.1–28.3°
μ = 0.17 mm^{-1}
T = 90 (1) K
Block, colourless
0.40 × 0.25 × 0.20 mm

Data collection

Bruker SMART APEX CCD diffractometer
ω scans
Absorption correction: empirical (*SADABS*; Sheldrick, 1996)
T_{min} = 0.797, T_{max} = 0.967
22 268 measured reflections
11 112 independent reflections
9973 reflections with $I > 2\sigma(I)$
R_{int} = 0.023
θ_{max} = 28.3°
$h = -17 \rightarrow 17$
$k = -18 \rightarrow 18$
$l = -17 \rightarrow 17$
342 standard reflections
frequency: 405 min
intensity decay: 0.5%

Refinement

Refinement on F^2
$R(F)$ = 0.051
$wR(F^2)$ = 0.137
S = 1.04
11 112 reflections
584 parameters
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.0937P)^2 + 0.2367P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max}$ = 0.004
$\Delta\rho_{max}$ = 0.85 e $Å^{-3}$
$\Delta\rho_{min}$ = −0.35 e $Å^{-3}$
Absolute structure: (Flack, 1983), 5087 Friedel pairs
Flack parameter = −0.06 (6)

Table 1
Hydrogen-bonding geometry (Å, °).

D—H···A	D—H	H···A	D···A	D—H···A
N_1—H1_1···O1_9	0.88	2.06	2.904 (2)	162
O1_9—H1_9···O1_11	0.85 (4)	2.05 (4)	2.793 (3)	147 (4)
O1_9—H2_9···N_6	0.89 (5)	2.02 (5)	2.896 (3)	168 (5)
O2_10—H1_10···O_2	0.96 (3)	1.88 (3)	2.838 (3)	175 (3)
CB_4—H30_4···O_2^{i}	0.95	2.25	3.175 (3)	163
CA_2—H16_2···O_8^{ii}	1.00	2.22	3.191 (3)	164
O2_10—H2_10···O_4^{iii}	0.92 (3)	1.89 (3)	2.799 (3)	177 (2)

Symmetry codes: (i) $2-x, y-\frac{1}{2}, 1-z$; (ii) $2-x, \frac{1}{2}+y, 2-z$; (iii) $2-x, \frac{1}{2}+y, 1-z$.

H atoms of the peptide and DMA molecules were calculated at ideal positions and were included in the refinement as riding atoms. Water H atoms were located in difference Fourier maps and were refined isotropically.

Data collection: *SMART* (Bruker, 1998); cell refinement: *SMART*; data reduction: *SAINT-Plus* (Bruker, 1998); program(s) used to solve structure: *SHELXD* (Sheldrick & Gould, 1996); program(s) used to refine structure: *SHELXL*97 (Sheldrick, 1997); molecular graphics: *PLATON* (Spek, 2001); software used to prepare material for publication: *PARST* (Nardelli, 1983).

Supplementary data for this paper are available from the IUCr electronic archives (Reference: OB1130). Services for accessing these data are described at the back of the journal.

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