Studies on *Rubia akane* (RA) derivatives. Part 10.[1] Backbone transformation of RA-VII, an antitumour cyclic hexapeptide, through thionation. X-Ray crystal structure of [Tyr-3-Ψ(CH_2NH)-Ala-4]RA-VII

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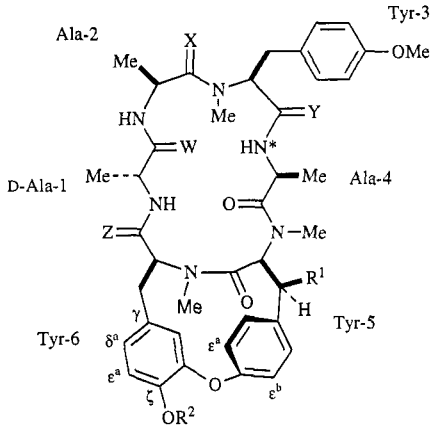
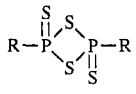
RA-VII 1 was thionated with Davy reagent methyl 6 or Davy reagent *p*-tolyl 7 to afford novel thionopeptides, [Tyr-6-Ψ(CS-NH)-D-Ala-1]RA-VII 8, [D-Ala-1-Ψ(CS-NH)-Ala-2; Tyr-3-Ψ(CS-NH)-Ala-4]RA-VII 9 and [Ala-2-Ψ(CS-NH)-Tyr-3; Tyr-3-Ψ(CS-NH)-Ala-4]RA-VII 10 in addition to known analogues [Tyr-3-Ψ(CS-NH)-Ala-4]RA-VII 4 and [Tyr-3-Ψ(CS-NH)-Ala-4; Tyr-6-Ψ(CS-NH)-D-Ala-1]RA-VII 5. Thionopeptide 4 was reduced with nickel borohydride to give reduced peptide 11. An X-ray analysis and an NMR study revealed that compound 11 adopts a different conformation within the 18-membered-ring moiety from the predominant solution conformation of peptide 1 and of thionopeptides 4, 5, 8–10, which would explain the loss in activity of compound 11.

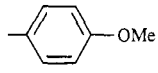
Introduction

RA-VII **1** is a bicyclic hexapeptide isolated from the plants *Rubia akane* and *R. cordifolia* (Rubiaceae)[2] and is closely related to bouvardin (NSC 259968, **2**) form *Bouvardia ternifolia*.[3] To date, fourteen RA congeners, RA-I–XIV, have been isolated from the same plants, and they are characterised by a strained 14-membered cycloisodityrosine structure.[4] Due to their promising antitumour activity with a unique mode of action, inhibition of protein synthesis through interaction with eukaryotic 80 S ribosomes,[5] peptide **1** is currently undergoing clinical trials in Japan as an anticancer agent.[6] It has been generally accepted that substitution of a thioamide for a peptide bond is considered to be an isosteric replacement due to their close structural similarities.[7] In some cases thioamide analogues show resistance against proteolytic enzymes and enhanced biological properties due to changes in their physical properties and pharmacokinetics.[8] In a previous communication, we have disclosed the thionation of compound **1** using two mole equivalents of Lawesson's reagent[9] [2,4-bis-(4-methoxyphenyl)-1,3,2λ^5,4λ^5-dithiadiphosphetane-2,4-dithione, LR **3**] in 1,4-dioxane which furnished [Tyr-3-Ψ(CS-NH)-Ala-4]-RA-VII **4** and [Tyr-3-Ψ(CS-NH)-Ala-4; Tyr-6-Ψ(CS-NH)-D-Ala-1]RA-VII **5** in yields of 80 and 3%, respectively, and that Tyr-3 monothionated analogues generally showed more potent cytotoxicity.[10] Since the contents of RAs and bouvardin in the plants are so small (~0.01% of dry roots for **1**; ~0.001% of dry aerial parts for **2**), ready access to a more potent analogue may constitute a possible solution to this supply problem. Also, since a thioamide shows different reactivity from a normal peptide toward reagents, thionation would enable selective transformation of a particular peptide bond. Although the 14-membered cycloisodityrosine moiety is proposed to be the pharmacophore for this class of antitumour agents,[11] little is known about the role of the 18-membered-ring moiety. To extend these lines of modifications in conjunction with further backbone modification of this important peptide in search of analogues which might express more promising biological properties or would provide information about structure/conformation–activity relationships, we further examined the thionation of peptide **1**.

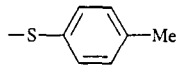


1 R^1 = H, R^2 = Me, W = X = Y = Z = O
2 R^1 = OH, R^2 = H, W = X = Y = Z = O
4 R^1 = H, R^2 = Me, W = X = Z = O, Y = S
5 R^1 = H, R^2 = Me, W = X = O, Y = Z = S
8 R^1 = H, R^2 = Me, W = X = Y = O, Z = S
9 R^1 = H, R^2 = Me, W = Y = S, X = Z = O
10 R^1 = H, R^2 = Me, W = Z = O, X = Y = S
11 R^1 = H, R^2 = Me, W = X = Z = O, Y = H_2
12 **11**: ⟶BH_3 at N*



3 R = 

6 R = SMe

7 R = 

Results and discussion

Although LR **3** is useful for the selective preparation of the Tyr-3 thioamides, it proved not to be suitable for obtaining further

Table 1 Thionation of compound **1** using Davy reagents[a]

Entry	Reagent	Reaction time (t/h)	Yield(%) 1	4	5	8	9	10
1	**6**	0.25	34	50	6.6	3.1	1.0	1.2
2	**6**	1.5	3.7	47	27	1.3	4.0	1.0
3	**6**	7	0	31	38	0	4.2	1.4
4	**7**	6	5.4	57	23	6.7	3.6	1.1
5	**7**	15	1.6	41	22	1.5	4.4	2.0

[a] The reaction was conducted in 1,4-dioxane at 50 °C.

thionated analogues.[10] Changes in the reaction conditions (solvent, temperature, reaction time or reagent stoichiometry) did not yield additional thionated compounds. We reasoned that this high selectivity is mainly due to the structural property of the substrate since the thionated positions (Tyr-3 and Tyr-6) are reactive secondary amide carbonyls not participating in the internal hydrogen bondings and due in part to the bulkiness of the reagent. Thus, alternative thionating reagents were sought, and Davy reagent methyl[12] [2,4-bis(methylthio)-1,3,2λ^5,4λ^5-dithiadiphosphetane-2,4-dithione, DRM 6] and Davy reagent *p*-tolyl[12] [2,4-bis-(4-methylphenythio)-1,3,2λ^5,4λ^5-dithiadiphosphetane-2,4-dithione, DRT 7] seemed to match our requirements. These reagents appear to be sterically more favourable in the transition state since the bulky aryl groups of LR are substituted by methylthio or *p*-tolylthio groups having long P–S bonds. When peptide **1** was treated with two mole equivalents of DRM **6** in 1,4-dioxane for 15 min, compounds **4** (50%), **5** (6.6%), recovered **1** (34%) were produced and novel thioamides **8**, **9** and **10** were obtained in yields of 3.1%, 1.0% and 1.2%, respectively (Table 1). These products were separated using preparative reversed-phase HPLC. High-resolution FAB mass spectra gave a molecular formula of $C_{41}H_{50}N_6O_8S$ for compound **8** and $C_{41}H_{50}N_6O_7S_2$ for compounds **9** and **10**, which indicated that compound **8** is a monothioamide and compounds **9** and **10** are dithioamides. The thionated positions were determined mainly by analysis of their ^{13}C NMR spectra. Since the resonances of thioamide carbonyls generally appear at very low field (δ ~200), complete assignments of all ^{13}C resonances would unambiguously establish the thioamide positions. The assignments of the ^1H and ^{13}C NMR chemical shifts for compounds **8**, **9** and **10** were accomplished using 2D NMR (COSY, NOESYPH,[13] HMQC[14] and HMBC[15]) techniques and the results are given in Tables 2 and 3. The assignments for compounds **1**, **4** and **5** are also given for comparison. The lowest field resonance of compound **8**, δ_C 203.07, was assigned to the thioamide at Tyr-6 residue, which determined the structure as [Tyr-6-Ψ(CS-NH)-D-Ala-1]RA-VII. In the same manner, compounds **9** (δ_C 196.85 and 204.62) and **10** (δ_C 194.29 and 196.46) were determined to be [D-Ala-1-Ψ(CS-NH)-Ala-2; Tyr-3-Ψ(CS-NH)-Ala-4]RA-VII and [Ala-2-Ψ(CS-NH)-Tyr-3; Tyr-3-Ψ(CS-NH)-Ala-4]RA-VII, respectively. Peptide **1** adopts two or three stable conformational states in solution,[16] and analogues **4**, **5**, **8**, **9** and **10** showed the same tendencies. The major conformer of each peptide in $CDCl_3$ shows NOESYPH correlations between the same protons (Fig. 1), and their ^1H NMR coupling constants are of similar value. This observation suggests that they adopt a very similar conformation in the major conformer although the conformer ratios varied among each peptide.† This major conformer is characterised by a type II β-turn structure centred to Ala-2 and Tyr-3 residues. The NOESYPH correlations

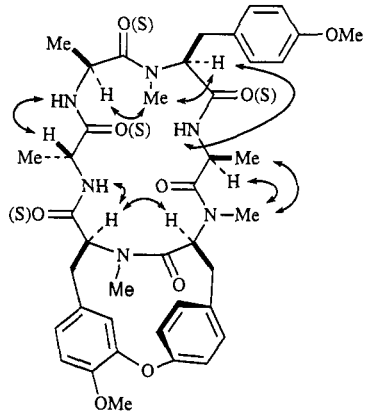


Fig. 1 Selected NOESYPH correlations commonly observed in the major conformer of compounds **1**, **4**, **5**, **8**–**10** in $CDCl_3$

between Tyr-3 N*Me* and Ala-4 N*H* and between Tyr-3 H^α and Ala-2 N*H*, and the small temperature coefficients[17] for Ala-4 N*H* in $(CD_3)_2SO$ ([$^2H_6$]DMSO) ($\Delta\delta/\Delta T$ = 0.2–0.8 × 10^{-3} ppm K^{-1}) supported this turn structure. Since these thioamides and peptide **1** adopt very similar conformations, comparison of their ^{13}C NMR chemical shifts leads to some generalities. The thioamide carbon and its α carbon resonances appear at ~24–32 ppm and ~6–7 ppm, respectively, to lower field than those for the parent peptide **1**. The α carbon of the residue adjacent to the C-terminal of the thioamide also shows a significant (~5–6 ppm) downfield shift. These tendencies appear to be additive because the α carbon of the Tyr-3 residue of the consecutive dithioamide **10** resonates at δ_C 78.83, which is 10.46 ppm down field from that of peptide **1**.

The product ratio of this reaction was greatly influenced by the reaction time. Elongation of the reaction time resulted in complete consumption of the substrate **1**, and the yields of products **4** and **8** were decreased. Probably both of these would be further thionated to compound **5** under the reaction conditions. However, the total yield of thioamides decreased as the reaction time increased, which is possibly due to the peptide-bond cleavage through formation of a thiazolethione.[18] Another thionating agent, DRT **7**, showed a similar tendency and produced the same products, but appears to be less reactive than DRM **6**.

The selective introduction,[10] into peptide **1**, of a single thioamide moiety in good yield using LR **3** afforded the opportunity of selectively obtaining the reduced peptide analogue [Tyr-3-Ψ(CH_2NH)-Ala-4]RA-VI **11**. This modification has been successfully applied for receptor agonists/antagonists[19] and enzyme inhibitors.[20] Furthermore, improvement of water solubility was anticipated through formation of an acid salt. When monothioamide **4** was reduced with nickel borohydride generated *in situ* from sodium borohydride and nickel(II) chloride hexahydrate,[21] two products, **11** and **12**, were obtained in yields of 19 and 54%, respectively. The more polar product **11** gave a molecular formula of $C_{41}H_{52}N_6O_8$ by a high-resolution FAB mass spectrum corresponding to the expected Tyr-3 deoxoanalogue, and the resonance of the

† Some cases have been reported where the thionated cyclic hexapeptide adopts very different conformations from those of the parent peptide.[26] However, for RAs, an anchoring effect of the rigid 14-membered cycloisodityrosine structure forces two β-turn structures centred onto the Ala-2 and Tyr-3 and onto the Tyr-5 and Tyr-6 residues.

Table 2 ^{1}H NMR chemical shift (δ) assignments and coupling constants (Hz, in parentheses) for the major conformer of compounds **1**, **4**, **5**, **8**–**12** in $CDCl_3$

	Position	**1**[a,b]	**4**[c]	**5**[c]	**8**[b]	**9**[b]	**10**[b]	**11**[b]	**12**[b]
D-Ala-1	α	4.36 (qd, 6.9, 6.7)	4.33 (qd, 7.0, 6.9)	4.82 (qd, 6.9, 6.7)	4.92 (qd, 6.7, 6.7)	4.51 (dq, 7.7, 6.5)	4.27 (dq, 7.0, 6.8)	4.31 (qd, 7.0, 6.5)	4.23 (qd, 7.0, 6.9)
	β	1.30 (d, 6.9)	1.29 (d, 7.0)	1.37 (d, 6.9)	1.38 (d, 6.7)	1.33 (d, 6.5)	1.30 (d, 7.0)	1.22 (d, 7.0)	1.29 (d, 7.0)
	NH	6.44 (d, 6.7)	6.35 (d, 6.9)	7.98 (d, 6.7)	7.98 (d, 6.7)	6.72 (d, 7.7)	6.34 (d, 6.8)	6.40 (d, 6.5)	6.37 (d, 6.9)
Ala-2	α	4.85 (dq, 8.4, 6.7)	4.94 (dq, 9.0, 6.9)	5.03 (dq, 9.5, 6.8)	4.97 (dq, 9.0, 6.7)	5.78 (dq, 9.0, 6.7)	5.23 (dq, 9.3, 6.6)	4.46 (dq, 9.0, 6.4)	5.01 (dq, 9.2, 6.8)
	β	1.35 (d, 6.7)	1.33 (d, 6.9)	1.33 (d, 6.8)	1.36 (d, 6.7)	1.42 (d, 6.7)	1.43 (d, 6.6)	0.86 (d, 6.4)	1.30 (d, 6.8)
	NH	6.35 (d, 8.4)	6.15 (d, 9.0)	6.16 (d, 9.5)	6.38 (d, 9.0)	8.06 (d, 9.0)	6.24 (d, 9.3)	7.48 (br d, 9.0)	6.16 (d, 9.2)
Tyr-3	α	3.58 (dd, 10.9, 5.0)	3.83 (dd, 10.9, 4.5)	3.83–3.92 (m)	3.59 (dd, 11.0, 5.1)	3.84 (dd, 11.2, 4.6)	4.20 (dd, 11.1, 4.0)	4.06 (m)	4.00 (m)
	β[a]	3.34 (dd, 14.0, 10.9)	3.52 (dd, 13.3, 10.9)	3.57 (dd, 14.7, 12.4)	3.36 (dd, 14.0, 11.0)	3.54 (dd, 13.7, 11.2)	4.27 (dd, 13.5, 11.1)		3.34 (dd, 13.2, 11.1)
	β[b]	3.38 (dd, 14.0, 5.0)	3.89 (dd, 13.3, 4.5)	3.83 3.92 (m)	3.39 (dd, 14.0, 5.1)	3.93 (dd, 13.7, 4.6)	3.90 (dd, 13.5, 4.0)	2.57–2.67 (m)	2.74 (dd, 13.2, 11.1)
	β′[a]								4.12 (dd, 11.2, 11.2)
	β′[b]							2.49 (dd, 10.8, 4.1)	2.62 (ddd, 11.2, 11.2, 3.8)
	δ	7.04 (d-like, 8.6)	7.06 (d-like, 8.6)	7.08 (d-like, 8.6)	7.05 (d-like, 8.6)	7.08 (d-like, 8.6)	7.14 (d-like, 8.6)	7.08 (d-like, 8.6)	7.09 (d-like, 8.6)
	ε	6.83 (d-like, 8.6)	6.83 (d-like, 8.6)	6.84 (d-like, 8.6)	6.84 (d-like, 8.6)	6.85 (d-like, 8.6)	6.84 (d-like, 8.6)	6.87 (d-like, 8.6)	6.85 (d-like, 8.6)
	NMe	2.86 (s)	2.85 (s)	2.86 (s)	2.84 (s)	2.84 (s)	3.09 (s)	2.86 (s)	2.82 (s)
	OMe	3.79 (s)	3.79 (s)	3.80 (s)	3.79 (s)	3.80 (s)	3.79 (s)	3.78 (s)	3.80 (s)
Ala-4	α	4.75 (dq, 7.6, 6.9)	5.31 (dq, 7.2, 6.7)	5.16 (qd, 6.7, 6.6)	4.67 (dq, 7.0, 6.6)	5.26 (dq, 7.0, 6.7)	5.39 (m)	3.42 (br q, 6.9)	3.78 (m)
	β	1.11 (d, 6.9)	1.24 (d, 6.7)	1.26 (d, 6.7)	1.22 (d, 6.6)	1.30 (d, 6.7)	1.24 (d, 6.8)	1.18 (d, 6.9)	1.13 (d, 6.7)
	NH	6.71 (d, 7.6)	8.51 (d, 7.2)	8.73 (d, 6.6)	6.79 (d, 7.0)	8.43 (d, 7.0)	8.18 (d, 7.4)	not detected	4.06 (m)
Tyr-5	α	5.41 (dd, 11.3, 3.0)	5.36 (dd, 11.4, 3.3)	5.34 (dd, 11.4, 3.4)	5.40 (dd, 11.3, 3.1)	5.27 (dd, 11.5, 3.5)	5.38 (m)	5.44 (dd, 11.5, 3.6)	5.32 (dd, 11.5, 3.7)
	β[a]	2.64 (dd, 11.4, 3.0)	2.64 (dd, 11.4, 3.3)	2.64 (dd, 11.4, 3.4)	2.61 (dd, 11.3, 3.1)	2.65 (dd, 11.5, 3.5)	2.63 (dd, 11.5, 3.2)	2.70 (dd, 11.5, 3.6)	2.85 (dd, 11.5, 3.7)
	β[b]	3.67 (dd, 11.4, 11.3)	3.64 (dd, 11.4, 11.4)	3.62 (dd, 11.4, 11.4)	3.66 (dd, 11.3, 11.3)	3.62 (dd, 11.5, 11.5)	3.66 (dd, 11.5, 11.5)	3.67 (dd, 11.5, 11.5)	3.58 (dd, 11.5, 11.5)
	δ[a]	7.26 (dd, 8.4, 2.2)	7.27 (dd, 8.4, 2.2)	7.28 (dd, 8.4, 2.2)	7.28 (dd, 8.5, 2.2)	7.29 (dd, 8.4, 2.2)	7.27 (dd, 8.4, 2.2)	7.29 (m)	7.27 (dd, 8.4, 2.2)
	δ[b]	7.42 (dd, 8.4, 2.2)	7.43 (dd, 8.3, 2.2)	7.48 (dd, 8.3, 2.2)	7.46 (dd, 8.4, 2.2)	7.49 (dd, 8.4, 2.2)	7.43 (dd, 8.4, 2.2)	7.54 (dd, 8.4, 2.2)	7.52 (dd, 8.4, 2.2)
	ε[a]	6.87 (dd, 8.4, 2.4)	6.88 (dd, 8.4, 2.3)	6.91 (dd, 8.4, 2.4)	6.89 (dd, 8.5, 2.4)	6.91 (dd, 8.3, 2.4)	6.88 (dd, 8.4, 2.4)	6.91 (dd, 8.4, 2.4)	6.88 (dd, 8.4, 2.4)
	ε[b]	7.20 (dd, 8.4, 2.4)	7.21 (dd, 8.3, 2.3)	7.23 (dd, 8.4, 2.4)	7.22 (dd, 8.4, 2.4)	7.22 (dd, 8.3, 2.4)	7.21 (dd, 8.4, 2.4)	7.29 (m)	7.22 (dd, 8.4, 2.4)
	NMe	3.13 (s)	3.19 (s)	3.16 (s)	3.08 (s)	3.23 (s)	3.18 (s)	3.12 (s)	3.25 (s)
Tyr-6	α	4.54 (dd, 12.0, 3.5)	4.50 (dd, 11.9, 3.7)	4.66 (dd, 11.7, 3.3)	4.74 (dd, 11.8, 3.4)	4.42 (m)	4.50 (dd, 12.0, 3.8)	4.58 (dd, 11.0, 4.8)	4.50 (dd, 11.9, 3.9)
	β[a]	3.10 (dd, 18.0, 12.0)	3.09 (dd, 18.0, 11.9)	3.12 (dd, 18.0, 11.7)	3.11 (dd, 18.0, 11.8)	3.10–3.06 (m)	3.10 (dd, 17.9, 12.0)	3.01–3.10 (m)	3.08 (dd, 18.1, 11.9)
	β[b]	2.95 (dd, 18.0, 3.5)	2.97 (dd, 18.0, 3.7)	2.83 (dd, 18.0, 3.3)	2.83 (dd, 18.0, 3.4)		2.97 (dd, 17.9, 3.8)		3.01 (dd, 18.1, 3.9)
	δ[a]	6.57 (dd, 8.3, 2.0)	6.58 (dd, 8.4, 2.1)	6.56 (dd, 8.4, 2.1)	6.55 (dd, 8.3, 1.8)	6.62 (dd, 8.4, 2.0)	6.58 (dd, 8.3, 2.1)	6.60 (dd, 8.3, 2.0)	6.58 (dd, 8.3, 2.0)
	δ[b]	4.34 (d, 2.0)	4.31 (d, 2.1)	4.33 (d, 2.1)	4.34 (d, 1.8)	4.34 (d, 2.0)	4.31 (d, 2.1)	4.35 (d, 2.0)	4.31 (d, 2.0)
	ε[a]	6.79 (d, 8.3)	6.80 (d, 8.4)	6.81 (d, 8.4)	6.80 (d, 8.3)	6.82 (d, 8.4)	6.80 (d, 8.3)	6.81 (d, 8.3)	6.80 (d, 8.3)
	NMe	2.69 (s)	2.66 (s)	2.74 (s)	2.75 (s)	2.61 (s)	2.67 (s)	2.65 (s)	2.65 (s)
	OMe	3.93 (s)	3.93 (s)	3.95 (s)	3.94 (s)	3.95 (s)	3.93 (s)	3.95 (s)	3.94 (s)

[a] Ref. 16. [b] 500 MHz. [c] 400 MHz.

Table 3 ^{13}C NMR chemical shift (δ) assignments for the major conformer of compounds **1**, **4**, **5**, **8**–**12** in $CDCl_3$

	Position	**1**[a,b]	**4**[c]	**5**[c]	**8**[b]	**9**[b]	**10**[b]	**11**[b]	**12**[b]
D-Ala-1	α	47.87	47.91	53.70	53.40	54.01	48.13	43.92	48.20
	β	20.67	20.75	18.07	18.11	23.64	20.59	20.66	20.61
	C=O (S)	172.73	171.94	171.35	171.53	204.62	171.79	171.66	171.63
Ala-2	α	44.56	44.35	44.45	44.41	50.40	49.04	48.31	45.18
	β	16.61	17.06	17.31	16.92	16.48	20.68	17.54	17.58
	C=O (S)	172.55	172.44	172.81	172.53	171.37	196.46	172.09	174.34
Tyr-3	α	68.37	74.24	75.03	68.43	74.05	78.83	59.37	64.25
	β	32.68	36.13	36.09	32.63	36.23	34.08	35.88	34.43
	γ	130.73	130.74	130.69	130.71	130.52	130.36	129.26	128.27
	δ	130.24	130.29	130.28	130.24	130.29	130.42	130.03	130.19
	ε	114.07	114.04	114.08	114.08	114.12	114.10	114.06	114.12
	ζ	158.45	158.49	158.56	158.47	158.58	158.64	158.52	158.73
	C=O (S, H_2)	168.01	197.54	197.21	167.74	196.85	194.29	49.46	50.30
	NMe	39.76	40.19	40.18	39.63	40.59	45.86	26.81	40.65
	OMe	55.26	55.26	55.30	55.28	55.28	55.26	55.31	55.30
Ala-4	α	46.43	51.39	51.57	46.62	51.51	51.55	54.35	58.73
	β	18.50	16.66	16.94	18.67	16.72	16.23	19.65	15.29
	C=O	171.77	171.27	171.23	171.65	171.16	171.33	173.62	172.24
Tyr-5	α	54.26	53.75	53.82	54.40	53.45	53.76	53.82	53.58
	β	36.99	36.97	37.46	37.50	36.97	36.95	36.87	36.84
	γ	135.16	134.69	134.74	135.20	134.49	134.61	134.82	134.58
	δ^a	132.79	132.89	132.76	132.63	132.99	132.91	132.87	132.92
	δ^b	130.98	131.08	131.34	131.33	130.96	131.05	131.21	131.31
	ε^a	124.24	124.36	124.28	124.09	124.44	124.38	124.42	124.37
	ε^b	125.91	125.91	126.01	125.97	125.96	125.89	125.89	125.97
	ζ	158.25	158.32	158.21	158.04	158.32	158.36	158.46	158.49
	C=O	169.33	169.55	169.83	169.56	170.01	169.42	170.50	170.37
	NMe	30.52	30.50	30.42	30.48	30.53	30.53	30.12	30.30
Tyr-6	α	57.39	57.53	64.13	63.96	57.84	57.52	57.92	57.78
	β	35.52	35.31	38.14	38.30	34.95	35.43	35.43	35.61
	γ	128.18	128.05	128.33	128.39	128.02	128.06	128.56	129.59
	δ^a	120.92	120.80	120.52	120.61	120.84	120.81	120.76	120.67
	δ^b	113.42	113.41	113.53	113.40	113.58	113.38	113.61	113.75
	ε^a	112.35	112.34	112.43	112.29	112.31	112.31	112.33	112.56
	ε^b	153.15	153.15	153.19	153.10	153.15	153.13	153.18	153.37
	ζ	146.54	146.50	146.48	146.44	146.48	146.50	146.40	146.58
	C=O (S)	170.71	170.40	202.81	203.07	168.81	170.46	171.21	170.61
	NMe	29.82	29.14	29.99	30.24	28.95	29.17	29.05	29.10
	OMe	56.18	56.17	56.22	56.17	56.21	56.16	56.19	56.28

[a] Ref. 16. [b] 125 MHz. [c] 100 MHz.

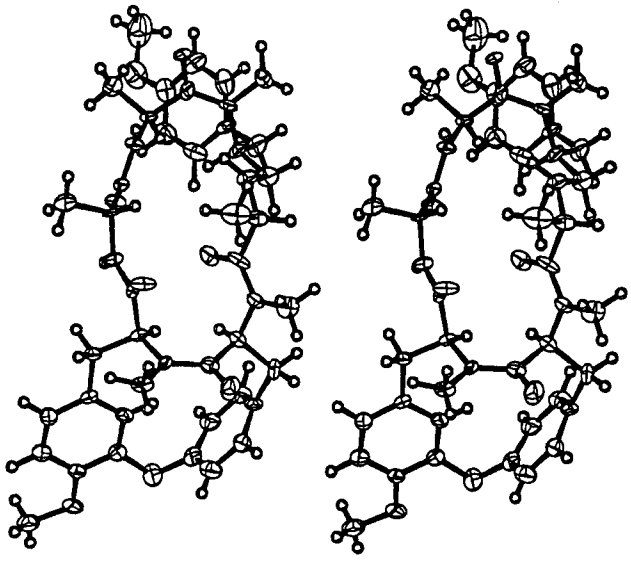


Fig. 2 A stereoscopic view of the crystal structure of compound **11**

Table 4 Selected conformational torsion angles (φ, ψ and ω; deg) of compound **11** in the solid state[a]

Residue		Torsion angle
D-Ala-1	φ	138(1)
	ψ	−160(1)
	ω	174(1)
Ala-2	φ	−142(1)
	ψ	95(1)
	ω	−6(2)
Tyr-3	φ	−125(1)
	ψ	55(1)
	ω	149.2(9)
Ala-4	φ	−65(1)
	ψ	162(1)
	ω	−179(1)
Tyr-5	φ	−108(1)
	ψ	103(1)
	ω	−7(2)
Tyr-6	φ	−86(1)
	ψ	170.8(9)
	ω	−169(1)

[a] The ESD values of X-ray torsion angles are given in parentheses.

reduced methylene carbon appeared at δ_C 49.46 in the ^{13}C NMR spectrum. To confirm absolute configurations of all stereogenic centres which might be epimerised under the reaction conditions, an X-ray diffraction analysis was conducted on compound **11**. A stereoscopic view is shown in Fig. 2, and the backbone dihedral angles are listed in Table 4. Although analogue **11** proved to retain the same absolute configurations at all amino acid residues and to possess a very similar 14-membered-ring structure, its 18-membered-ring conformation is different from that in peptide **1**. The most different features from peptide **1** and thioamides **4**, **5**, **8**–**10** in this crystal structure are the adoption of a *cis* amide bond between the Ala-2 and Tyr-3 residues and the absence of internal hydrogen bonding between D-Ala-1 C*O* and Ala-4 N*H* (O · · · N at 4.64 Å). Compound **11** appears to possess very similar structure to this crystal structure in its major conformer

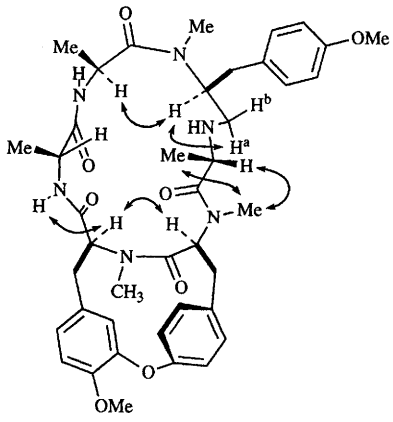


Fig. 3 Selected ROESY correlations for the major conformer of compound **11** in $CDCl_3$

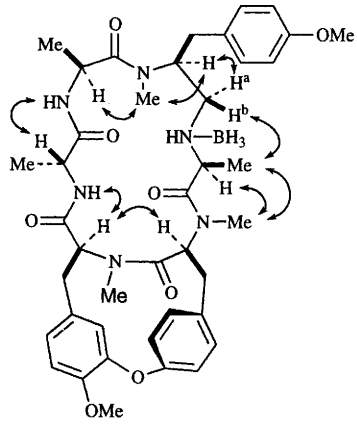


Fig. 4 Selected ROESY correlations for the major conformer of adduct **12** in $CDCl_3$

(>90% population) in $CDCl_3$ solution. The ROESY[22] correlations observed for peptide **11** are shown in Fig. 3. The correlations between Ala-2 H^{α} and Tyr-3 H^{α} and between Tyr-5 H^{α} and Tyr-6 H^{α} indicated that peptide **11** possesses *cis* amide bonds between Ala-2 and Tyr-3 and between Tyr-5 and Tyr-6 residues. The most pertinent calculated dihedral angles using the modified Karplus equation‡,[23] for the N–H–C^{α}–H plane of D-Ala-1 and Ala-2 residues are −138° and 162°, respectively, which are in good agreement with the values obtained from X-ray analysis (−149° and 154°). One of the values calculated for the dihedral angle of $C^{\beta'}$–H^{a}(*pro*S)–C^{α}–H plane of Tyr-3 residue using the original Karplus equation,§[24] is 43° and is also similar to the value (61°) obtained from the X-ray data. The temperature coefficients for D-Ala-1 N*H* and Ala-2 N*H* in [$^2H_6$]DMSO are -4.8×10^{-3} and -5.0×10^{-3} ppm K^{-1}, respectively, which show that these amide hydrogens are exposed to the solvent and are not participating in the internal hydrogen bonding.[17] Although peptide **1** and its thioamides have small populations of a conformer having a *cis* amide bond between the Ala-2 and Tyr-3 residues,[16] these crystal and solution structures of peptide **11** are distinguishable from it by the absence of the internal hydrogen bonding between D-Ala-1 C*O* and Ala-4 N*H* which stabilises a type-VI β-turn structure of the conformer, and by the difference of the backbone dihedrals. These geometrical changes in compound **11** compared with peptide **1** and the parent thioamide **4** are attributed to the change in the almost planar Tyr-3–Ala-4 amide bond (ω = −168° for **1**[16]) to a CH_2–NH single (σ) bond (ω = 149.2°) with tetrahedral sp^3 carbon and nitrogen atoms, which enhances the flexibility of the molecule through reduction of the rotational barrier and reduces the acidity of NH engaged in the internal hydrogen bonding.

The less polar product **12** gave the same number of resonances in its ^{13}C NMR spectrum (including DEPT experiment) as did compound **11** but showed a relative molecular mass of 769.3 [M − H] in the negative-ion FAB mass spectrum. This relative molecular mass corresponds to a molecular formula of $C_{41}H_{55}BN_6O_8$, and compound **12** was considered to be a borane complex of peptide **11**. The characteristic IR band at 2320 cm^{-1} (ν_{B-H}) supported this composition. Compound **12** is fairly stable in $CHCl_3$. No discernible change was observed in the ^1H NMR spectra when the $CDCl_3$ solution of compound **12** was kept for 29 days at 37 °C. However, complex **12** gradually decomposed to peptide **11** in methanol solution ($t_{\frac{1}{2}}$ = 72 h at 37 °C). Compound **12** adopts almost only one (~95%) conformational state in $CDCl_3$ solution. The ROESY correlations (Fig. 4) between Ala-2 H^{α} and Tyr-3 N*Me* and between Tyr-3 N*Me* and Tyr-3 H^{α} show the presence of a *trans* amide bond between Ala-2 and Tyr-3 residues, and complex **12** appears to adopt a similar conformation to the major solution conformation of peptides **1** and **2**, which is considered to be at least in part responsible for its activity.[25]

‡ $^3J_{NH-CH} = 6.4 \cos^2\theta - 1.4 \cos\theta + 1.9$ (Hz).
§ ^3J 4.22–0.5 cos θ + 4.5 cos 2θ (Hz).

Table 5 Cytotoxicity of compound **1** and analogues against P-388 murine leukaemia cells

Compound	IC_{50}(μgcm^{-3})
1	0.001 3
4	0.000 58
5	0.001 7
8	0.002 6
9	0.004 4
10	0.001 3
11	>10
12	1.0

Prepared analogues were evaluated using P-388 murine leukaemia cells, and the results are summarised in Table 5. All the thionated analogues retained potent cytotoxicity, which is attributed to their structural/conformational similarities to peptide **1**. Although monothionation at the Tyr-3 residue generally enhances the activity,[10] further thionation at either D-Ala-1, Ala-2 or Tyr-6 residue or monothionation at Tyr-6 residue posed no or a rather detrimental effect on the activity. Although analogue **11** incorporates the pharnacophore unit, a 14-membered cycloisodityrosine moiety,[11] it showed no activity. The complete loss in activity of compound **11** is possibly due to the significant conformational change within the 18-membered-ring moiety. Although it gradually decomposed to compound **11** under the assay conditions (2 days incubation at 37 °C in aqueous media), the borane complex **12**, and although possessing a similar conformation to peptide **1**, showed only weak activity. These results emphasise the importance of the 18-membered-ring structure for full expression of its biological activity.

Experimental

Organic solutions, dried over Na_2SO_4, were evaporated under an aspirator vacuum with a rotary evaporator. High-pressure liquid chromatography (HPLC) was performed with a Shimadzu LC-6AD system. Medium-pressure liquid chromatography (MPLC) was performed with a Kusano C.I.G. system. Mps were taken on a Yanagimoto melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 digital polarimeter and are recorded in units of 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded on a JASCO A-302 or Hitachi 260–10 spectrophotometer. NMR spectra were

measured on a Bruker AM-400, AM-500 or JEOL A-500 spectrometer. ^{1}H Chemical shifts are referenced in $CDCl_3$ to residual $CHCl_3$ (δ 7.26); ^{13}C chemical shifts are referenced to the solvent ($CDCl_3$; δ_C 77.03). *J*-Values are given in Hz. Mass spectra were taken using a VG AutoSpecE spectrometer.

Thionation of peptide 1 using Davy reagents

Typical procedure. A solution of compound **1** (101.9 mg, 0.132 mmol) in 1,4-dioxane (3 cm^3) was treated with DRM **6** (75.3 mg, 0.265 mmol) at 50 °C for 15 min. Water (2 cm^3) was added to the solution, and the whole was stirred at room temperature for 30 min. The mixture was extracted with chloroform. The extract was washed successively with saturated aq. $NaHCO_3$ and brine, dried, and then evaporated to dryness. Chromatography on alumina with dichloromethane–ethyl acetate–methanol (12:2:1) as the eluent yielded a mixture of thioamides, which was separated by HPLC [Inertsil PREP-ODS (20 × 250 mm, 10μm), water–methanol (3:7), 10 cm^3 min^{-1}] to provide recovered substrate **1** (34.7 mg, 34%; t_R 17.3 min) and thioamides **8** (3.2 mg, 3.1%; t_R 25.8 min), **4** (51.9 mg, 50%; t_R 38.7 min), **5** (7.0 mg, 6.6%; t_R 63.1 min), **10** (1.3 mg, 1.2%; t_R 96.3 min) and **9** (1.1 mg, 1.0%; t_R 114.3 min).

Compound **8**: amorphous solid, mp 230–233 °C; $[\alpha]_D^{25}$ −120.6 (*c* 0.18, $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3400, 2950, 1640, 1510, 1445, 1410, 1265, 1250, 1220, 1130, 1100 and 1030; HR-FAB-MS [Found: (M + H), 787.3509. $C_{41}H_{51}N_6O_8S$ requires (M + H), 787.3489].

Compound **9**: amorphous solid, mp 187–191 °C; $[\alpha]_D^{25}$ −270.0 (*c* 0.60, $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3340, 2940, 1640, 1515, 1505, 1440, 1410, 1265, 1250, 1205, 1130, 1095 and 1030; HR-FAB-MS [Found: (M + H), 803.3280. $C_{41}H_{51}N_6O_7S_2$ requires (M + H), 803.3261].

Compound **10**: amorphous solid, mp 185–192 °C; $[\alpha]_D^{25}$ −280.0 (*c* 0.18, $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3310, 2930, 1660, 1635, 1510, 1440, 1405, 1260, 1245, 1090 and 1030; HR-FAB-MS [Found: (M + H), 803.3237. $C_{41}H_{51}N_6O_7S_2$ requires (M + H), 803.3261].

Nickel borohydride reduction of thioamide 4

To a solution of thioamide **4** (60 mg, 0.076 mmol) and nickel(II) chloride hexahydrate (145 mg, 0.61 mmol) in tetrahydrofuran (3 cm^3) was added sodium borohydride (69 mg, 1.82 mmol), and the mixture was stirred at room temperature for 1 h. The mixture was passed through a Celite pad, and the solvent was evaporated off *in vacuo*. The residue was dissolved in dichloromethane and washed with brine, dried, and then evaporated to dryness. MPLC (SiO_2) with dichloromethane–ethyl acetate–methanol (12:2:1) yielded complex **12** (31.8 mg, 54%), and successive elution with dichloromethane–methanol (5:1) yielded peptide **11** (10.8 mg, 19%).

Compound **11**: prisms, mp 287–289 °C (from acetonitrile), $[\alpha]_D^{25}$ −133.2 (*c* 0.34, $CHCl_3$); ν_{max}(KBr)/cm^{-1} 3390, 2940, 1660, 1625, 1510, 1500, 1440, 1410, 1260, 1240, 1205, 1130 and 1030; HR-EI-MS [Found: M, 756.3866. $C_{41}H_{52}N_6O_8$: requires M, 756.3847].

Compound **12**: amorphous solid, mp 265–269 °C; $[\alpha]_D^{26}$ −122.1 (*c* 0.10, $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 3380, 2920, 2320, 1660, 1445, 1135, 1095 and 1030; negative-FAB-MS: *m*/*z* 769.3 ([M − H]).

X-Ray crystal structure determination of compound 11

Crystal data. $C_{41}H_{52}N_6O_8$, M = 756.90. Orthorhombic, a = 12.695(2), b = 37.83(1), c = 8.781(4) Å, V = 4217(2) Å^3. Cu-Kα (λ = 1.541 78 Å), space group $P2_12_12$ (#18), Z = 4, D_x = 1.19 g cm^{-3}. Prismatic. Crystal dimensions 0.25 × 0.20 × 0.43 mm, μ(Cu-Kα) 6.8 cm^{-1}.

Data collection. Data were collected on a Rigaku AFC5S diffractometer at a temperature of 173 ± 1 K using the ω–2θ scan technique to a maximum 2θ-value of 119.6°. Scans of (1.10 + 0.30 tan θ)° were made at a speed of 32.0° min^{-1} (in omega). The weak reflections [$I < 10.0\ \sigma(I)$] were rescanned (maximum of 3 scans) and the counts were accumulated to ensure good counting statistics.

Data reduction. A total of 3553 reflections was collected. The intensities of three representative reflections were measured after every 150 reflections. No decay correction was applied. An empirical absorption correction using the program DIFABS[27] was applied which resulted in transmission factors ranging from 0.86 to 1.06. The data were corrected for Lorentz and polarisation effects.

Structure solution and refinement. The structure was solved by direct methods (SHELXS86)[28] and expanded using Fourier techniques.[29] The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were introduced at idealised positions but were not refined. The final cycle of full-matrix least-squares refinement was based on 2339 observed reflections [$I > 3.00\ \sigma(I)$] and 496 variable parameters and converged with unweighted and weighted agreement factors of: $R = \Sigma||F_o| - |F_c||/\Sigma|F_o| = 0.065$, $R_w = \{\Sigma w(|F_o| - |F_c|)^2/\Sigma wF_o^2\}^{\frac{1}{2}} = 0.067$.

The standard deviation of an observation of unit weight was 3.19. The weighting scheme was based on counting statistics and included a factor (p = 0.004) to 'downweight' the intense reflections. Plots of $\Sigma w(|F_o| - |F_c|)^2$ *versus* $|F_o|$, reflection order in data collection, sin θ/λ and various classes of indices showed no unusual trends. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.35 and −0.28 e^- Å^{-3}, respectively.

Neutral-atom scattering factors were taken from ref. 30*a*. Anomalous dispersion effects were included in F_{calc};[31] the values for $\Delta f'$ and $\Delta f''$ were taken from ref. 30*b*. The values for the mass attenuation coefficients were taken from ref. 30*c*.

Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre.¶

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¶ For details of the deposition scheme, see 'Instructions for Authors', *J. Chem. Soc., Perkin Trans. 1*, 1996, Issue 1. Any request to the CCDC for this material should quote the full literature citation and the reference number 207/7.

References

1 Part 9, Y. Hitotsuyanagi, S. Lee, K. Takeya and H. Itokawa, *J. Chem. Soc., Chem. Commun.*, in the press (5/05846E).
2 H. Itokawa, K. Takeya, K. Mihara, N. Mori, T. Hamanaka, T. Sonobe and Y. Iitaka, *Chem. Pharm. Bull.*, 1983, **31**, 1424; H. Itokawa, K. Takeya, N. Mori, T. Hamanaka, T. Sonobe and K. Mihara, *Chem. Pharm. Bull.*, 1984, **32**, 284.
3 S. D. Jolad, J. J. Hoffmann, S. J. Torrance, R. M. Wiedhopf, J. R. Cole, S. K. Arota, R. B. Bates, R. L. Gargiulo and G. R. Kriek, *J. Am. Chem. Soc.*, 1977, **99**, 8040.
4 H. Itokawa and K. Takeya, *Heterocycles*, 1993, **35**, 1467.
5 M. Zalacaín, E. Zaera, D. Vázquez and A. Jiménez, *FEBS Lett.*, 1982, **148**, 95.
6 H. Majima, S. Tsukagoshi, H. Furue, M. Suminaga, K. Sakamoto, R. Wakabayashi, S. Kishino, H. Niitani, A. Murata, A. Genma, N. Nukariya, K. Uematsu, T. Furuta, M. Kurihara, F. Yoshida, S. Isomura, T. Takemoto, M. Hirashima, T. Izumi, I. Nakao, Y. Ohashi, K. Ito and R. Asai, *Jpn. J. Cancer Chemother.*, 1993, **20**, 67; F. Yoshida, R. Asai, H. Majima, S. Tsukagoshi, H. Furue, M. Suminaga, K. Sakamoto, H. Niitani, A. Murata, M. Kurihara, T. Izumi, I. Nakao, Y. Ohashi and K. Ito, *Jpn. J. Cancer Chemother.*, 1994, **21**, 199.
7 T. F. M. La Cour, H. A. S. Hansen, K. Clausen and S.-O. Lawesson, *Int. J. Pept. Protein Res.*, 1983, **22**, 509; M. Kruszynski, G. Kupryszewski, U. Ragnarsson, M. Alexandrova, V. Strbak, M. C.

Tonon and H. Vaudry, *Experientia*, 1985, **41**, 1576; L. Maziak, G. Lajoie and B. Belleau, *J. Am. Chem. Soc.*, 1986, **108**, 182; V. N. Balaji, S. Profeta, Jr. and S. W. Dietrich, *Biochem. Biophys. Res. Commun.*, 1987, **145**, 834; D. B. Sherman and A. F. Spatola, *J. Am. Chem. Soc.*, 1990, **112**, 433.
8 P. Campbell and N. T. Nashed, *J. Am. Chem. Soc.*, 1982, **104**, 5221; P. A. Bartlett, K. L. Spear and N. E. Jacobsen, *Biochemistry*, 1982, **21**, 1608; R. E. Beattie, D. T. Elmore, C. H. Williams and D. J. S. Guthrie, *Biochem. J.*, 1987, **245**, 285; K. L. Foje and R. P. Hanzlik, *Biochim. Biophys. Acta*, 1994, **1201**, 447.
9 M. Thorsen, B. Yde, U. Pedersen, K. Clausen and S.-O. Lawesson, *Tetrahedron*, 1983, **39**, 3429.
10 Y. Hitotsuyanagi, J. Suzuki, Y. Matsumoto, K. Takeya and H. Itokawa, *J. Chem. Soc., Perkin Trans. 1*, 1994, 1887.
11 D. L. Boger, D. Yohannes and J. B. Meyers, Jr., *J. Org. Chem.*, 1992, **57**, 1319; D. L. Boger, D. Yohannes, J. Zhou and M. A. Patane, *J. Am. Chem. Soc.*, 1993, **115**, 3420.
12 H. Davy, *J. Chem. Soc., Chem. Commun.*, 1982, 457; H. Davy and P. Metzner, *J. Chem. Res. (S)*, 1985, 272; B. Yde, N. M. Yousif, U. Pedersen, I. Thomsen and S.-O. Lawesson, *Tetrahedron*, 1984, **40**, 2047.
13 G. Bodenhausen, H. Koger and R. R. Ernst, *J. Magn. Reson.*, 1984, **58**, 370.
14 L. Müler, *J. Am. Chem. Soc.*, 1979, **101**, 4481.
15 A. Bax and M. F. Summers *J. Am. Chem. Soc.*, 1986, **108**, 2093.
16 H. Morita, K. Kondo, Y. Hitotsuyanagi, K. Takeya, H. Itokawa, N. Tomioka, A. Itai and Y. Iitaka, *Tetrahedron*, 1991, **47**, 2757.
17 M. Iqbal and P. Balaram, *Biopolymers*, 1982, **21**, 1427.
18 P. Wipf, C. Jenny and H. Heimgartner, *Helv. Chim. Acta*, 1987, **70**, 1001.
19 D. H. Coy, P. Heinz-Erian, N.-Y. Jiang, Y. Sasaki, J. Taylor, J.-P. Moreau, W. T. Wolfrey, J. D. Gardner and R. T. Jensen, *J. Biol. Chem.*, 1988, **263**, 5056; M. Rodriguez, J.-P. Bali, R. Magous, B. Castro and J. Martinez, *Int. J. Pept. Protein Res.*, 1989, **27**, 293; D. Lugrin, F. Vecchini, S. Doulut, M. Rodriguez, J. Martinez and P. Kitabgi, *Eur. J. Pharmacol.*, 1991, **205**, 191; T. P. Davis, S. Crowell, J. Taylor, D. L. Clark, D. Coy, J. Staley and T. W. Moody, *Peptides*, 1992, **13**, 401.
20 M. Szelke, B. Leckie, A. Hallet, D. M. Jones, J. Sueiras, B. Atrash and A. F. Lever, *Nature*, 1982, **299**, 555.
21 F. S. Guziec, Jr. and L. M. Wasmund, *Tetrahedron Lett.*, 1990, **31**, 23.
22 A. A. Bothner-By, R. L. Stephens, J. Lee, C. D. Warren and R. W. Jeanloz, *J. Am. Chem. Soc.*, 1984, **106**, 811.
23 A. Pardi, M. Billeter and K. Wüthrich, *J. Mol. Biol.*, 1984, **180**, 741.
24 M. Karplus, *J. Am. Chem. Soc.*, 1963, **85**, 2870.
25 H. Itokawa, K. Kondo, Y. Hitotsuyanagi, M. Isomura and K. Takeya, *Chem. Pharm. Bull.*, 1993, **41**, 1402.
26 H. Kessler, A. Geyer, H. Matter and M. Köck, *Int. J. Pept. Protein Res.*, 1992, **40** 25; H. Kessler, H. Matter, A. Geyer, H.-J. Diehl, M. Köck, G. Kurz, F. R. Opperdoes, M. Callens and R. K. Wierenga, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 328.
27 N. Walker and D. Stuart, *Acta Crystallogr., Sect. A*, 1983, **39**, 158.
28 G. M. Sheldrick, C. Kruger and R. Goddard, Oxford University Press, Oxford 1985, pp. 175–189.
29 P. T. Beurskens, G. Admiraal, G. Beurskens, W. P. Bosman, S. Garcia-Granda, R. O. Gould. J. M. M. Smith and C. Smykalla, Technical Report of the Crystallography Laboratory, University of Nijmegen, The Netherlands, 1992.
30 *International Tables for Crystallography*, ed. A. J. C. Wilson, Kluwer Academic, Dordrecht, Boston and London, 1992, vol. C, (*a*) Table 6.1; (*b*) Table 4.2.6.8; (*c*) Table 4.2.4.3.
31 J. A. Ibers and W. C. Hamilton, *Acta Crystallogr.*, 1964, **17**, 781.

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