Crystal Structures of Actinomycin D and Actinomycin Z_3**

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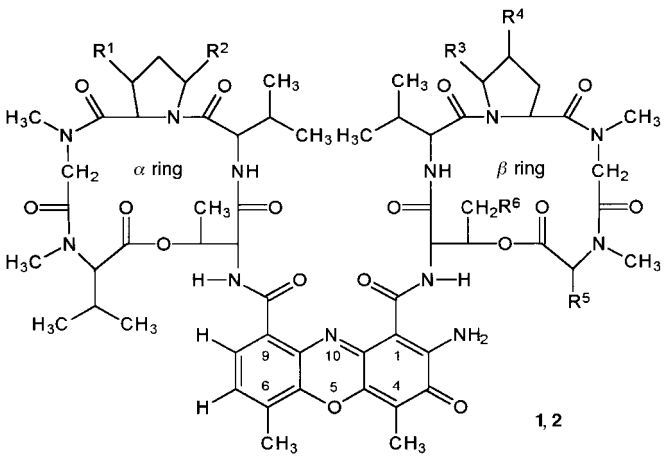
In memory of Hans Brockmann

Actinomycins are antibiotically and cytostatically highly effective chromopeptides isolated from *Streptomyces* cultures, of which over thirty native and many synthetic variants are known.[1] All possess the same chromophore, a 2-aminophenoxazin-3-one system, which is coupled by acid amides to two identical or different pentapeptide lactone (depsipeptide) rings (*iso*- and *aniso*-actinomycins, respectively). Actinomycin D (= C_1, **1**; Scheme 1) has been most intensively investigated; in spite of its high toxicity it is employed inter alia for therapy of rare diseases such as the Wilms tumor. The mode of action of the actinomycins is particularly interesting from the standpoint of molecular biology.



Scheme 1. Actinomycin D (= C_1, **1**): R^1 = H, R^2 = H, R^3 = H, R^4 = H, R^5 = $CH(CH_3)_2$, R^6 = H; actinomycin Z_3 (**2**): R^1 = OH, R^2 = CH_3, R^3 = CH_3, R^4 = O, R^5 = CH_3, R^6 = Cl.

This involves—as proposed already in 1968[2] and later verified by NMR spectroscopy[3] and X-ray structural analysis of the actinomycin/deoxyguanosine[4] and DNA octamer complexes[5, 6]—an insertion (intercalation) of the chromophore between two guanine/cytosine (GC) base pairs of the double-stranded helical DNA. The two depsipeptide rings fit into the minor groove of the DNA, and each forms two hydrogen bonds between the NH and CO groups of the threonine units and the neighbouring nitrogen atoms of the guanine bases; in addition there are two hydrogen bonds from the 2-amino group to a phosphate oxygen atom of the DNA. The hydrophobic amino acids of both depsipeptide rings (Pro, Sar, MeVal) lie on the GC base pairs on either side of the insertion position of the chromophore, whereby adjacent hydrophobic adenine/thymine base pairs have a stabilizing influence.[7] These amino acid/DNA contacts protect the essential hydrogen bonds against competition reactions with nearby water molecules. As a result of intercalation of the actinomycin the spatial structure of the DNA is slightly distorted, which leads to an obstruction of the DNA-dependent RNA synthesis and DNA replication; one molecule in 1000 base pairs suffices for an effect.[2] The activity of the antibiotic is extremely sensitive to chemical structural changes; it is reduced to zero by, for example, the opening of one depsipeptide ring or the replacement of the methyl groups on the chromophore by *tert*-butyl or CF_3 groups.[8]

Attempts to solve the crystal structure of an uncomplexed actinomycin have been made since about 1960. Bachmann and Müller[9] obtained one rhombohedral and two monoclinic forms of actinomycin C_3,[10] which, however, appeared to be twinned about a pseudo-twofold axis. Palmer et al.[11] found a further probably monoclinic crystal form with a similar twinning; a structural model could not be obtained. The first real crystal structure determination of actinomycin D occurred in 1988.[12] Ginell et al. found the same crystal form as Palmer et al. had previously, but were able to solve the structure in the space group *P*1. However, as a result of the very weak data (maximum resolution 1.1 Å) as well as the twinning, it was only possible to refine the structure as a rigid group to an *R* value of 14%.

We have now been able to obtain for the first time untwinned crystals that diffracted to atomic resolution of two actinomycins, actinomycin D (**1**) and actinomycin Z_3 (**2**); the constitution of the latter was only recently elucidated by Shigamatsu et al.[13] Of particular interest here are the conformations of the α- and β-depsipeptide rings in the uncomplexed state, their intramolecular interactions, and the packing of the actinomycin molecules in the crystal lattice, including their hydrophobic and hydrophilic contacts to one another and to solvent molecules.

Actinomycin D (**1**) crystallized out of ethyl acetate/methanol as orange-red single crystals with the triclinic space group *P*1.[14] The unit cell of **1**, referred to subsequently as **1***, contains three independent molecules of **1** (A, B, and C) together with one methanol and seven ethyl acetate molecules. By way of example, Figure 1 shows three views of molecule C. The geometry of A, B, and C is almost identical, whereby A and B, with a root mean square deviation (rmsd) of only 0.29 Å, fit one another better than A and C (0.71 Å) or B and C (0.78 Å). Each molecule possesses approximately C_2 symmetry; the pseudo-twofold axis runs through the line joining O5 and N10. Only the unsymmetrical substitution at C2 and C3 of the chromophore prevents the presence of a genuine twofold axis. In each of the three molecules, two antiparallel hydrogen bonds are formed between the amide nitrogen atom of the D-valine residue of the α ring and the carbonyl oxygen atom of the D-valine residue of the β ring, and vice versa (Table 1). These hydrogen bonds stabilize the

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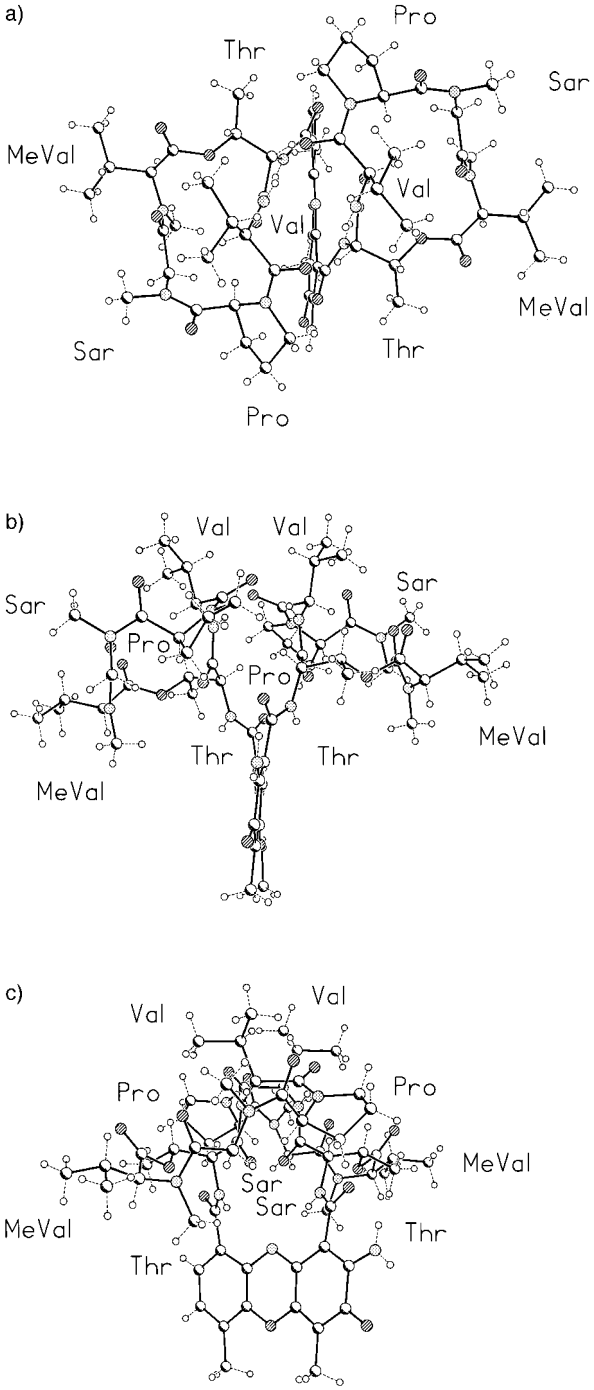

Figure 1. Three-dimensional structure of **1** (molecule C in the unit cell **1***). a) Top view: the β-depsipeptide ring is shown on the right, and the α ring on the left; the 2-amino group of the chromophore that lies under the depsipeptides points downwards. b) Side view: the β ring lies again on the right; the 2-amino group extends forwards. Underneath on the flat chromophore that is then inserted between the GC base pairs of the DNA one can recognize the methyl groups that are important for the stability of the complex. c) Front view: the chromophore (bottom) is bent slightly by the voluminous and conformationally stiff depsipeptide rings (see also b). Thr = L-threonine, Val = D-valine, Pro = L-proline, Sar = sarcosine, MeVal = *N*-methyl-L-valine. The dotted atoms are N, and the shaded are O atoms.

"A" conformation[15] of the depsipeptide rings present here, and are thus consistent with all previously observed conformations in the solid state[4–6, 16] and in solution.[17] The superposition of the six depsipeptide rings from A – C gives a maximum rmsd of only 0.29 Å.

Table 1. Distances [Å] in several important intramolecular hydrogen bonds in **1*** and **2***.

	A	B	C	D	E
$N(Val_\beta)\cdots O(Val_\alpha)$	2.91	2.93	2.93	3.00	2.90
$N(Val_\alpha)\cdots O(Val_\beta)$	2.87	2.87	2.86	2.88	2.91
$N2\cdots O(C1-CO)$	2.74	2.77	2.62	2.68	2.68
$N(Thr_\beta)\cdots N10$	–	–	2.76	2.81	2.81
$N(Thr_\alpha)\cdots N10$	–	–	2.83	2.96	2.94

Molecules A and B form a dimer across a pseudo-twofold axis. The chromophores are stacked parallel to one another with a shortest distance of 3.43 Å (Figure 2). The amide

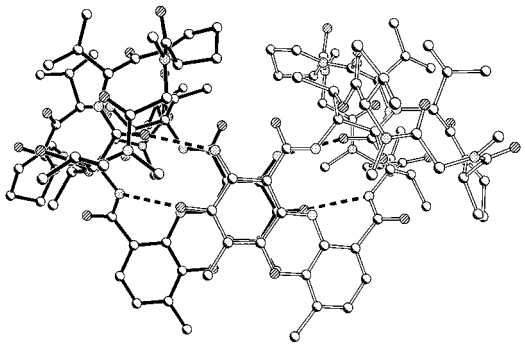

Figure 2. The dimer of the molecules A and B in **1***. Molecule A is indicated by solid bonds, and B by open bonds. The twofold axis runs perpendicular between the chromophore systems that are stacked with their quinone rings overlapping. The dashed bonds correspond to the hydrogen bonds.

nitrogen and carbonyl oxygen atoms of the threonine residue in the α-depsipeptide rings of A and B form two antiparallel hydrogen bonds to O3 and N2, respectively, of the chromophore of the corresponding other molecule ($N(Thr_\alpha)\cdots O3$ 3.07 and 3.13 Å, respectively; $N2\cdots O(Thr_\alpha)$ 2.94 and 2.96 Å, respectively). Since, however, in the **1**/DNA complex these groups are bonding partners for the GC base pairs, the above dimerization is clearly only stable in the free actinomycin D.[18] Molecule C shows no signs of dimerization; there is only a weak interaction between the 2-amino group of the chromophore and the carbonyl oxygen atom of the β-ring proline of molecule B of a neighboring asymmetric unit ($N2\cdots O(Pro_\beta)$ 3.03 Å). This CO group also forms a hydrogen bond to a methanol molecule ($O(Me\cdots O(Pro_\beta)$ 2.83 Å). The ethyl acetate molecules that are distributed throughout the cell do not enter into such interactions.

Actinomycin Z_3 (**2**) was recently isolated in pure form as an important component of the actinomycin Z mixture, and the amino acid sequence of its α- and β-depsipeptide rings was elucidated.[13] It is—together with actinomycin Z_5, which was first characterized at the same time—the first native chlorine-containing actinomycin, and displays the very rare 4-chloro-L-threonine (ClThr) instead of L-threonine in the β-depsipeptide ring. Furthermore the β ring contains *N*-methyl-L-alanine (MeAla) and *cis*-5-methyl-4-oxo-L-proline (MOPro) instead of *N*-methyl-L-valine and L-proline, respectively, and

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the α ring contains *trans*-3-hydroxy-*cis*-5-methyl-L-proline (HMPro) instead of L-proline.

Red single crystals of **2** suitable for X-ray analysis were obtained from benzene and have space group $P2_12_12_1$. Unlike the structure of **1** there are only two independent molecules (D and E) in the asymmetric unit,[14] subsequently denoted by **2***. In addition, one water and twenty benzene molecules could be located in a difference Fourier synthesis; thus, this structure has a solvent content of 37.4 % (**1***: 14.7 %). Figure 3 shows the three-dimensional structure of molecule D in **2***.

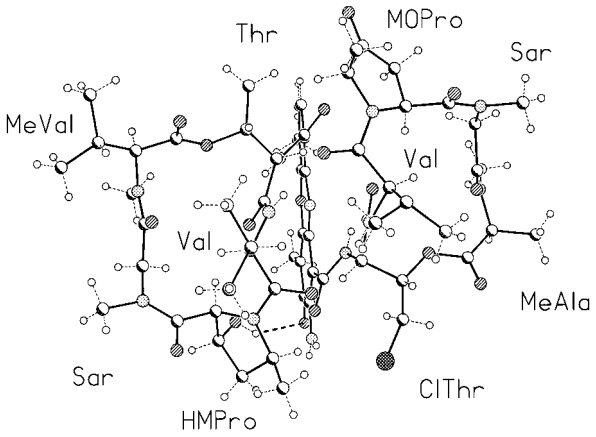

Figure 3. Three-dimensional structure of **2** in the unit cell **2***. The representation shown here with the β-depsipeptide ring on the right corresponds to that in Figure 2a. The thick dashed bond (bottom) marks the extra hydrogen bond from 3-O(HMPro) to 1-CO on the chromophore. MeAla = *N*-methyl-L-alanine, MOPro = *cis*-5-methyl-4-oxo-L-proline, HMPro = *trans*-3-hydroxy-*cis*-5-methyl-L-proline, ClThr = 4-chloro-L-threonine.

Although **2** also possesses pseudo-C_2 symmetry with the axis along the line connecting O5 and N10, it is much more strongly distorted than **1** as a result of the presence of different amino acids in the α- and β-depsipeptide rings. Both D-valine residues in **2** form, analogous in those in **1**, the reciprocal hydrogen bonds that stabilize the "A" conformation of the depsipeptide rings (Table 1). In addition, one finds an intramolecular hydrogen bond between the hydroxyl group of the hydroxymethylproline (α ring) and the 1-carbonyl oxygen atom on the chromophore (Figure 3; O(HMPro$_\alpha$)$\cdots$ O(1-CO) 2.82 Å in both D and E). The four depsipeptide rings in **2*** are again almost identical (maximum rmsd 0.33 Å); their conformations also correspond closely to those of the six depsipeptide rings in **1*** (rmsd 0.32 Å; Figure 4).

The independent molecules D and E in **2*** are also nearly identical (rmsd 0.24 Å). Their superposition onto molecule A in **1*** shows that the overall conformations of the two actinomycins Z_3 and D are comparable (rmsd 0.56 and 0.50 Å, respectively). A dimerization as in **1*** is, however, not to be found in **2***. Molecules D and E form a chain structure along the crystallographic *a* axis that is stabilized by intermolecular hydrogen bonds. The water molecule that had been located in the difference Fourier synthesis forms two hydrogen bonds, one to the carbonyl oxygen atom of the chlorothreonine (O(H_2O)$\cdots$O(ClThr) 2.86 Å), and the other

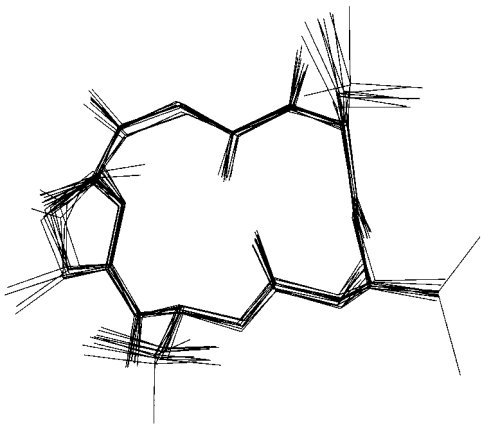

Figure 4. Least-squares fit of all ten depsipeptide rings of **1*** and **2*** on top of one another.

to the 9-carbonyl oxygen atom of the chromophore in molecule D (O(H_2O)$\cdots$O(9-C) 2.99 Å).

Kamitori and Takusagawa[6] showed in their study of an actinomycin/DNA complex that the depsipeptide rings can adapt their conformations by rotation about the bonds C1 – CO and C9 – CO in the chromophore, depending on the width of the minor groove. Thus, it is interesting to compare the corresponding torsion angles C2-C1-CO-N(Thr$_\alpha$) and C8-C9-CO-N(Thr$_\alpha$) (Table 2). One can recognize two trends here: The torsion angle in the β ring is larger than that in the α ring,

Table 2. Summary of the C2-C1-CO-N(Thr) and C8-C9-CO-N(Thr) torsion angles [°] in **1*** and **2*** (molecules A– E) as well as in the actinomycin/desoxyguanosine complex (F)[4] and actinomycin/DNA complex (G).[5]

	A	B	C	D	E	F	G
α ring	131.8	115.1	141.2	128.4	130.7	101	89.7
β ring	141.8	140.2	151.7	144.9	150.2	112	91.3

and from the free actinomycin (molecules C, D, E) through the dimer (A, B) to the complexed actinomycin (F, G), these angles become smaller.

In free actinomycin the threonine nitrogen atoms are forced towards each other in the direction of the chromophore plane, so that they have weak intramolecular interactions with N10 of the chromophore (Table 1). In the dimer the N(Thr) atoms are rotated further out of the chromophore plane (torsion angles 131.8 and 115.1°, respectively), the intramolecular saturation is lost,[19] and the α-ring amide nitrogen atom can take part in *inter*molecular interactions. In the actinomycin/DNA complex (torsion angles about 90°) the amide nitrogen atoms stick right out of the chromophore plane and can now make the essential hydrogen bonds to the guanine residue of the DNA. The rotational freedom of the conformationally otherwise relatively inflexible depsipeptide rings about the C1 – CO and C9 – CO axes, together with that about the N – C bonds of the threonine residues,[20] makes it possible for the actinomycin molecule to reorientate the α- and β-depsipeptide rings so far that they can be fitted optimally into the minor groove. When the depsipeptide rings are structurally appreciably different, as in the *aniso*-actinomycin Z_3 (**2**), the

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resulting lack of symmetry of the whole molecule (Figure 3) should make the adaption and intercalation process more difficult. If then amino acid variants that are more hydrophilic weaken the important hydrophobic contacts of the depsipeptide rings with the adenine/thymine base pairs of the DNA, the reduction in the biological activity observed for actinomycins of the Z type becomes plausible.

Several factors played decisive roles in the final elucidation of the precise structure of the actinomycins: First, the growing of untwinned crystals, which was successful here for the actinomycins for the first time, was an essential precondition. The collection of high-resolution data sets was achieved by the use of low temperatures, an intensive X-ray beam, and an area detector, and led to much more detailed insights into the three-dimensional structures of the molecules. Finally the development of new ab initio methods of solving structures, as they are implemented in the program SHELXD,[21] now makes the automated determination of structures with more than about 200 independent non-hydrogen atoms possible. The solution of these two structures on a Pentium processor required several hours. A solution of the structure of **1*** with 314 non-hydrogen atoms in the asymmetric unit with traditional direct methods would have been unthinkable. The determination of the structures of further actinomycins and other interesting "large small molecules" can thus be anticipated in the near future.

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[14] a) Crystallographic data for **1***: $3\,C_{62}H_{86}N_{12}O_{16} + 7\,C_4H_8O_2 + CH_3OH$, $M_r = 4410$, triclinic, space group $P1$, $a = 15.737(5)$, $b = 15.887(5)$, $c = 25.156(10)$ Å, $\alpha = 85.93(1)$, $\beta = 86.19$, $\gamma = 69.86(1)$, $V = 5883.6(4)$ Å^3, $Z = 1$, $\rho_{calcd} = 1.246$ M gm^{-3}, $F(000) = 2364$, $= 1.54178$ Å, $T = 133$ K, $\mu(Cu_{K\alpha}) = 0.76$ mm^{-1}, min./max. transmission 0.7251/0.9276, crystal dimensions $0.45 \times 0.45 \times 0.1$ mm^3, $2.72^\circ \le 2\Theta \le 108.16^\circ$. Of 65014 measured data, 14126 were independent ($R_{int} = 0.0444$, Friedel mates merged), refinement: 2875 parameters, 3689 restraints, $R1 = \Sigma\,|F_o - F_c|\,/\Sigma F_o = 0.0545$ ($I > 2\sigma(I)$) and $wR2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma wF_o^4]^{1/2} = 0.1477$ (all data); min./max. difference electron density $-0.47/0.71$ e Å^{-3}.[14c] b) Crystallographic data for **2***: $2\,C_{62}H_{83}N_{12}O_{18}Cl + 20\,C_6H_6 + H_2O$, $M_r = 4219.8$, othorhombic, space group $P2_12_12_1$, $a = 14.803(3)$, $b = 24.780(5)$, $c = 65.069$ (10) Å, $V = 23865(8)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.174$ M gm^{-3}, $F(000) = 9000$, $\lambda = 1.54178$ Å, $T = 173$ K, $\mu(Cu_{K\alpha}) = 0.838$ mm^{-1}, min./max. transmission 0.7304/0.7871, crystal dimensions $0.4 \times 0.3 \times 0.3$ mm^3, $2.72^\circ \le 2\Theta \le 108.16^\circ$. Of 112696 data, 15653 were independent ($R_{int} = 0.0602$, Friedel mates merged). Refinement: 3024 parameters, 5470 restraints, $R1 = \Sigma\,|F_o - F_c|\,/\Sigma F_o = 0.0666$ ($I > 2\sigma(I)$), $wR2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma wF_o^4]^{1/2} = 0.1820$ (all data); min./max. difference electron density: $-0.343/0.357$ e Å^{-3}. The chlorine atom in molecule E is disordered over two positions with occupancies of 0.85:0.15. Four of the twenty benzene molecules are also disordered over two positions and refined to occupancies of 0.55:0.45, 0.45:0.55, 0.57:0.43, and 0.63:0.37. Intensity data for **1*** and **2*** were recorded for shock-cooled crystals mounted in oil drops[22] on a three-circle diffractometer with a multiwire proportional area detector with φ and ω scans. The reflections were integrated with the program SAINT. All data were corrected semi-empirically for systematic errors such as absorption. The structures were solved by direct methods (SHELXD)[21] and refined against F^2 by least squares.[23] All non-hydrogen atoms could be refined anisotropically. The hydrogen atoms were placed in geometrically ideal positions and refined with a riding model, in which the methyl groups (except those of solvent molecules) could rotate about their local threefold axes. All disorders could be resolved and refined anisotropically with the help of distance and *ADP* restraints. The crystallographic data of the structures described in this publication have been deposited with the Protein Data Bank (PDB), Brookhaven National Laboratory, under the codes 1A7Y und 1A7Z.

[15] In the "A" form the peptide bonds from L-threonine up to L-methylvaline adopt the conformations *trans*, *cis*, *cis*, *trans*.[17]

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[18] A different type of dimerization was deduced from NMR data for aqueous solutions of actinomycin D: The chromophores overlap more strongly, but no intermolecular hydrogen bonds are formed in this model.[24]

[19] The distances between the threonine nitrogen atoms and N10 (2.88 Å in A, 2.94 Å in B) are in the range for hydrogen bonds, but this is not true of the N(Thr)-H(Thr) $\cdots$ N10 angles.

[20] The corresponding torsion angles H-N(Thr)-C(Thr)-H lie in the range of 146.6° to 155.2° in all five molecules.

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