X-ray Crystal Structure of 28-*O*-Methylrapamycin complexed with FKBP12: Is the Cyclohexyl Moiety Part of the Effector Domain of Rapamycin?

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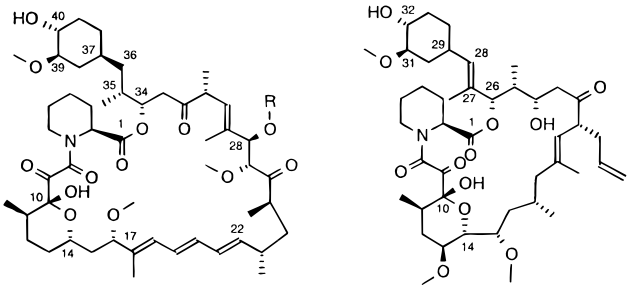
Abstract: The X-ray crystal structure of 28-*O*-methylrapamycin **2** bound to FKBP12 is described. This rapamycin analogue binds to FKBP12 with an affinity comparable to that of rapamycin, but its immunosupressive activity is reduced by a factor of 1000. The atomic structure of the complex formed by FKBP12 and 28-*O*-methylrapamycin is compared with those of the FKBP12−rapamycin and FKBP12−FK506 complexes. The steric 28-*O*-methyl group induces a dramatic shift in the orientation of the cyclohexyl moiety, which is now in a position similar to the one observed for the cyclohexyl subunit in the FKBP12−FK506 complex. The conformation of the macrocyclic part of the molecule remains unchanged. As a consequence of 28-*O*-methylation and the resulting modified orientation taken by the cyclohexyl subunit, two intermolecular hydrogen bonds between the ligand and the binding protein are lost in comparison to the FKBP12−rapamycin complex. The affinity for FKBP12 is not significantly affected by these structural changes, but the immunosuppressive activity is greatly reduced, suggesting a critical role for the cyclohexyl ring in the interaction of the FKBP12−rapamycin complex with its molecular target.

Introduction

Rapamycin **1** (Figure 1) was described for the first time in 1975 as an antifungal agent, isolated from a strain of *Streptomyces hygroscopicus* originating from Easter Island (Rapa Nui).[1] Despite the fact that the immunosuppressive activity of rapamycin was recognized as early as 1977,[2] this property of the macrolide has only recently attracted widespread interest in view of its potential therapeutic use in the prevention of transplant rejection.[3] Rapamycin **1** shares a common structural motif with another potent immunosuppressant, FK506 (**3**).[4] These homologous regions, covering the pipecolyl ester, the hemiketal-masked tricarbonyl region, and the pyranosyl ring, have been shown by X-ray crystallography to be responsible for the binding of rapamycin[5ac] and FK506[5b,c] to their common intracellular receptor FKBP12[6] and have therefore been termed the "binding domains" of these molecules.[7] The remaining part of each macrolide represents its "effector domain" [7] and is critical for its immunosuppressive activity. The peptidyl−prolyl isomerase (PPIase) activity of FKBP12 is inhibited by binding of FK506[8] or rapamycin,[9] but this inhibition does not correlate with immunosuppressive activity.[10,11] Moreover, it was found that rapamycin and FK506 exert their immunosuppressive activity by different mechanisms, FK506 inhibiting the production of IL-2 and other cytokines by T-cells, and rapamycin interfering at a later stage of T-cell activation by inhibiting the proliferative response to IL-2 and other growth factors.[12] In specific biological assays, rapamycin and FK506 can even antagonize each other.[12] Taken together, these observations clearly high-



1: Rapamycin (R = H)
2: 28-O-Methyl-rapamycin (R = Me)

3: FK506

Figure 1. Chemical structures of rapamycin (**1**), 28-*O*-methylrapamycin (**2**) and FK506.

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light the importance of the distinct effector domains of these macrolides, and they have been designated as "dual domain" inhibitors.[7,13] Both macrolides bind to FKBP12 through their common binding domain, and the resulting complexes become the actual immunosuppressive species, which, in virtue of their distinct exposed effector domains, interact with distinct intracellular targets.[7,13] The FKBP12−FK506 complex binds to and inhibits the serine−threonine phosphatase calcineurin, thereby disrupting the signal transduction pathway emanating from the T-cell receptor which leads to the transcription of the gene for the cytokine IL-2.[14] Recently a protein of hitherto unknown function, which is bound by the FKBP12−rapamycin complex, termed FRAP[15a] (RAFT,[15b] RAPT,[15c] mTOR[15d]) has been described by several groups. Indirect evidence indicates that this could be the relevant target for the immunosuppressive activity of rapamycin.[15a] Not much is known about the precise structural features required for the antiproliferative action of rapamycin. The pipecolinyl ring is deeply buried in FKBP12. The pyranose ring, the cyclohexyl ring and the region extending from C34 to C28 (Figure 1) are in close contact with the protein, with the three hydroxyls at positions 10, 28, and 40 involved in hydrogen bonds. The region from C16 to C27, including the characteristic C17−C22 triene, is exposed[5a,c] and therefore available for interaction with FRAP. The triene appears to be critical, as demonstrated by a derivative modified in that region, which is still a potent PPIase inhibitor, but which is not immunosuppressive.[16] We report herein an X-ray crystallographic analysis of 28-*O*-methylrapamycin (**2**) bound to FKBP12 showing, as the sole major change with respect to the FKBP12−rapamycin complex, a dramatic shift in the orientation of the cyclohexyl ring, correlating with a loss of immunosuppressive activity.

Results and Discussion

In the course of a program aimed at establishing a structure−activity relationship for rapamycin derivatives we wanted to probe the role of the C28-hydroxyl and prepared 28-*O*-methylrapamycin (**2**).[17,18] In a competitive FKBP12-binding assay **2** inhibited binding of FK506 with an IC_{50} of 1.8 ± 0.6 nM (rapamycin: IC_{50} 1.1 ± 0.4 nM),[19] indicating that methylation of the C28-hydroxyl did not significantly affect the affinity toward FKBP12. This result came as a surprise, as the C28-hydroxyl had been shown to make a hydrogen bond with the Glu-54 main chain carbonyl.[5a,c] Even if this single hydrogen bond could be anticipated to contribute relatively little to the overall binding energy, it was nevertheless expected that, due to the close proximity of the C28-OH to the protein backbone, *O*-methylation at this position would result in severe steric interaction with FKBP12 and thus in strongly reduced binding. The immunosuppressive activity of **2** was assessed *in vitro* in a two-way allogeneic mixed lymphocyte reaction (MLR);[20] the IC_{50} of 164 nM was more than 1300-fold higher than that observed for rapamycin in the same assay (IC_{50} 0.12 nM). In order to gain insight into the structural basis for these results, 28-*O*-methylrapamycin (**2**) was cocrystallized with human recombinant FKBP12. A high-resolution X-ray analysis (details of data collection and refinement statistics are shown in Table 1) of the resulting complex yielded a structure with an estimated accuracy of coordinates of 0.25 Å (from a Luzzati plot). The conformation of the ligand was clearly defined, as can be seen from the $2F_o - F_c$ electron density contoured at 1σ (data 8−2.1 Å) depicted in Figure 2.

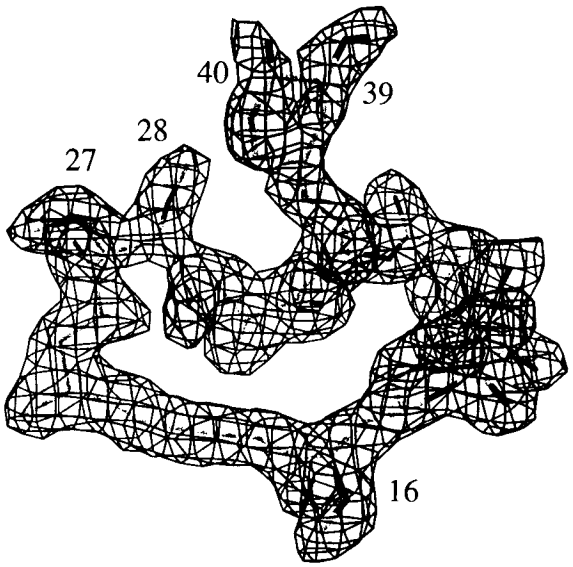

Figure 2. Final $2F_o - F_c$ electron density map (calculated using all data from 8 to 2.1 Å) contoured at 1σ for 28-*O*-methylrapamycin bound to FKBP12. The locations of carbon atoms C16, C27, C28, C39, and C40 (cf. Figure 1) are nearby to indicated numbers.

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(17) 28-*O*-Methylrapamycin (**2**) was prepared in three steps from rapamycin **1**: (a) selective monosilylation of C40-OH with 1.1 equiv of TBSOTf and 2.2 equiv of 2,6-lutidine in methylene chloride at 0 °C (83%); (b) methylation of C28-OH using 3 equiv of trimethyloxonium tetrafluoroborate in the presence of 5 equiv proton sponge in methylene chloride at room temperature (60%) (ref 18); (c) desilylation in 18:1 acetonitrile−HF·pyridine at 0 °C (51%).

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(19) In this assay, FK506 coupled to BSA is used to coat microtiter well plates. Biotinylated human FKBP12 is allowed to bind to the immobilized FK506 in the absence (negative control) or presence of a test sample. Bound biotinylated FKBP12 is assessed by incubation with a streptavidin−alkaline phosphatase conjugate, followed by incubation with *p*-nitrophenyl phosphate as a substrate and determination of the OD at 405 nM. This competitive binding assay is conceptually similar to the ones used by other authors to determine the affinity of FK506[8] and rapamycin[9] to FKBP12. The affinity of rapamycin to FKBP12 as determined in our assay is consistent with data reported in the literature.[9]

(20) Spleen cells from two genetically different donors are mixed and cultivated for 4 days in the absence or presence of a serially diluted test sample and rapamycin as a standard. ^{3}H-thymidine is then added, the cells are harvested after another 16 h incubation period, and ^{3}H-thymidine-incorporation, a measure for cell proliferation, is determined.

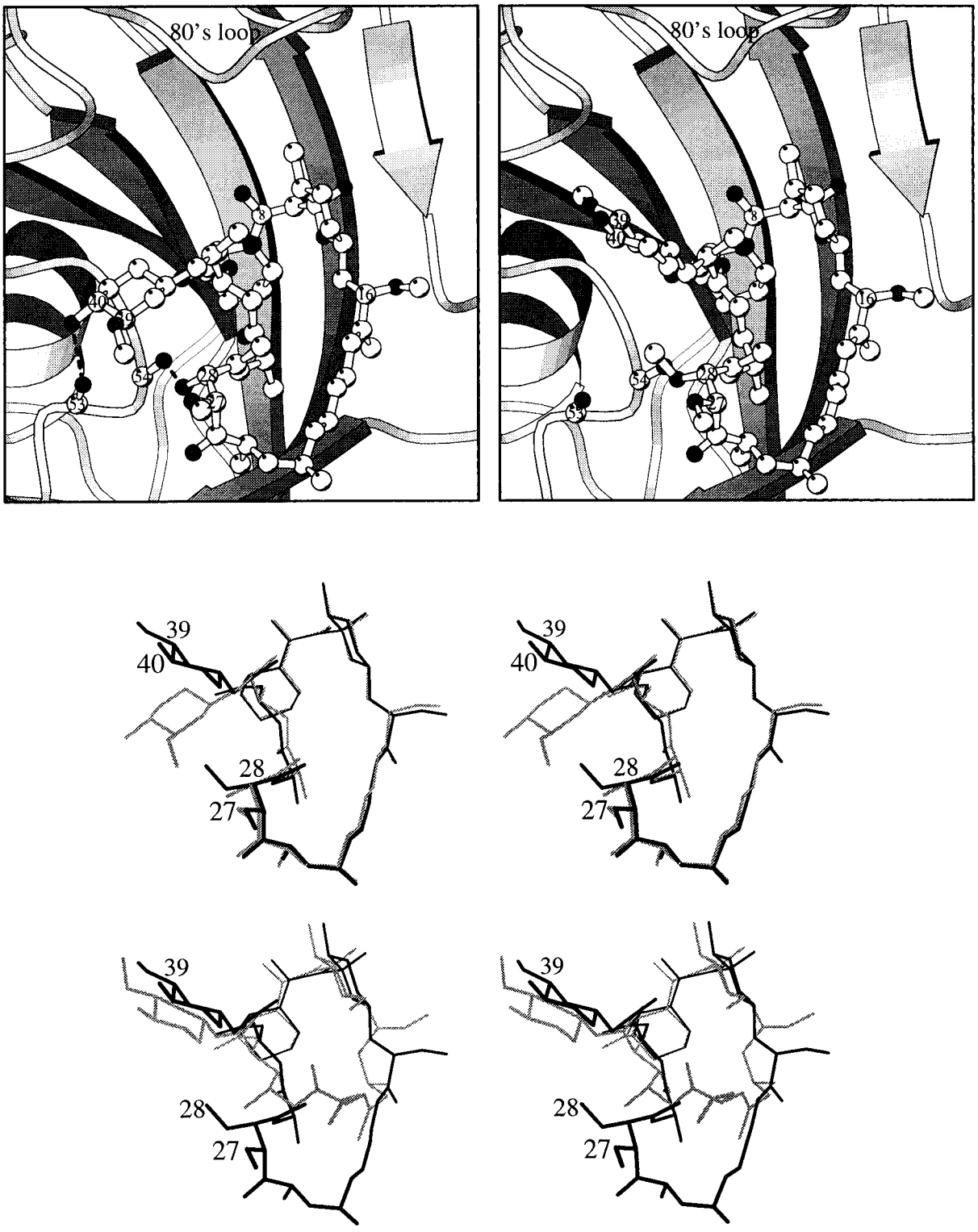

Figure 3. (A, top left) The conformation of rapamycin (ball-and-stick model) when bound to FKBP12 (ribbon diagram), with the main-chain carbonyl groups of residues Gln-53 and Glu-54 of FKBP12 also shown as ball-and-stick models. The intermolecular hydrogen bonds between C40-hydroxyl and Gln53-CO, and between C28-hydroxyl and Glu54-CO, respectively, are indicated as dashed lines. (B, top right) The conformation of 28-*O*-methylrapamycin when bound to FKBP12. A comparison with part A shows that two intermolecular hydrogen bonds have been lost and that the cyclohexyl moiety has dramatically changed its position. Also access to the 80's loop of FKBP12 "from the left" is now hindered by the novel position of the cyclohexyl moiety. (C, middle) Superposition of 28-*O*-methylrapamycin (black) and rapamycin (gray), both when bound to FKBP12, using a least-squares transformation calulated on Cα atoms of FKBP12 (yielding a rms deviation of 0.18 Å for Cα atoms of FKBP12). (D, bottom) Superposition of 28-*O*-methylrapamycin (black) and FK506 (gray), both when bound to FKBP12, using a least-squares transformation calculated on Cα atoms of FKBP12 (yielding a rms deviation of 0.62 Å for Cα atoms of FKBP12).

The protein components of the complexes FKBP12−rapamycin (FKBP12−**1**) and FKBP12−28-*O*-methylrapamycin (FKBP12−**2**) are almost identical, with a root-mean-square difference of 0.18 Å for the protein Cα atoms after least-squares superposition of these atoms. The largest backbone shifts occur in the 50's loop, for residues Lys-52, Gln-53, and Glu-54, attaining values of 0.7, 0.7, and 0.5 Å for the respective backbone carbonyl−oxygen atoms. In the complex FKBP12−**1**

the 50's loop makes hydrogen bonds to the ligand, namely between Gln-53 main chain carbonyl and 40-OH (distance 2.8 Å) and between Glu-54 main chain carbonyl and 28-OH (distance 2.8 Å), as depicted in Figure 3A. Formation of the latter hydrogen bond is of course not possible in the complex FKBP12−**2**, and through movements of the two oxygen atoms of Glu-54 main chain carbonyl (by 0.5 Å) and 28-O (by 0.7 Å) their distance is increased from 2.8 Å to 3.5 Å. The methyl group of the 28-*O*-methyl moiety points away from the protein, toward the cyclohexyl portion C35−C42 (Figure 1), with the torsion angle (C27,C28,28-*O*,28-*O*-methyl) being 127°. The macrocyclic C1−C34 part of the ligand remains almost unchanged with respect to the conformation it adopts in bound rapamycin, as can be seen by comparison of Figures 3A and 3B, as well as by examination of the overlay of bound **2** and bound **1** (Figure 3C). The most striking difference concerns the cyclohexylethyl moiety C35−C42 in **2**, which is dramatically shifted in comparison to rapamycin. If the cyclohexyl portion did not move, i.e. had an identical position as in rapamycin, there would be severe steric interaction with the 28-*O*-methyl, e.g. the distance between the carbon atom of 28-*O*-methyl and C39 would be 2.0 Å. In order to avoid this unfavorable interaction, the cyclohexyl subunit moves into a new orientation. Although such a change could have been expected, its magnitude was not anticipated. The cyclohexylethyl moiety C35−C42 now adopts a conformation which closely resembles the position of the analogous cyclohexylethenyl subunit C27−C34 (Figure 1) of bound FK506 **3** (Figure 3D). The ring changes its position by simply rotating around the C35,C36 bond (by a value of roughly 120°), this being the major structural change in going from FKBP12−**1** to FKBP12−**2** (Figure 3C). More precisely, the torsional angle (C34,C35,C36,C37) around the C35,C36 bond for bound **1** has a value of −65°, whereas for bound **2** the value is −171°. This latter value is close to the 175° found for the corresponding torsional angle (C26,C27,C28,C29) around the C27,C28 double bond in FK506 bound to FKBP12. Figures 3C,D show superpositions of **2** with **1** and FK506, respectively.

It is unlikely that the loss of immunosuppressive activity observed for 28-*O*-methylrapamycin (**2**) is simply due to direct steric interactions of the newly introduced methyl group with the target protein. The C28-*O*-methyl group is certainly not more sterically demanding than the cyclohexyl moiety which occupies the same region around the 50's loop in the FKBP12−rapamycin complex (Figures 3A,B,C). It is more likely that the presence of the 28-*O*-methyl indirectly causes loss of immunosuppressive potency by inducing an unfavorable orientation of the cyclohexyl moiety. In the FKBP12−rapamycin complex, the cyclohexyl is in close contact with the 50's loop through hydrogen bond formation between C40-OH and the Gln-53 main chain carbonyl. This leaves space open in the valley flanked by the 50's and 80's loops for a potential interaction with a target protein (Figure 3A). The cyclohexyl ring of **2** bound to FKBP12 is located right in the middle between the 50's and 80's loops and therefore could hinder a proper interaction between the immunophilin−ligand complex and its target (Figure 3B). Purely steric factors could then be responsible for the loss of biological activity. Alternatively, the target might have to recognize the precise structure of the cyclohexyl ring, or part of it, with its particular functionality in a defined position. This would imply that the cyclohexyl portion is actually part of the effector domain of rapamycin. We are presently not able to determine precisely which mechanism is operating. Refinement of our hypotheses will necessitate the synthesis of further derivatives modified in the cyclohexyl region. Work along these lines is currently underway in our laboratories and the results of this study will be reported in due time.

Table 1. Protein Crystallography Data Collection Statistics and Refinement Results

space group	$P2_12_12_1$
cell constants	
a (Å)	45.5
b (Å)	49.7
c (Å)	55.3
no. of complexes per asymm unit	1
% solvent	48
data collection statistics	
resolution (Å)	15−2.1
no. of reflcns measd	15899
no. of unique reflcns	6946
R_{sym} (on intensities) overall	9.1
R_{sym} for 2.15−2.1 Å	29.0
% complete overall	90
% complete for 2.15−2.1 Å	84
no. of refined non-H atoms	
protein	832
ligand	66
solvent	91
R factor (no σ cutoff)	18.7
R factor for 2.15−2.1 Å	27.4
rms bond dev (Å)	0.016
rms angle dev (deg)	3.2
rms improper torsion dev (deg)	1.3
av *B* for protein (Å^2)	23.3
av *B* for ligand (Å^2)	19.1

Conclusion

The X-ray crystal structure of **2** bound to FKBP12 was determined in order to provide a structural rationale for its biological activity. It was found that, while the macrocyclic part of the ligand is unchanged with respect to the FKBP12−rapamycin complex, the cyclohexyl moiety is dramatically shifted. This structural change does not affect binding to FKBP12, in spite of the disruption of two hydrogen bonds, but results in significantly reduced immunosuppressive activity. An unfavorable steric interaction of the cyclohexyl ring in its new position with the target of the FKBP12−rapamycin complex might explain this loss of activity. Alternatively, the cyclohexylethyl side chain could exert a functional role in target recognition, thus making it part of rapamycin's effector domain.

Experimental Procedures

Protein Crystallography. Crystallization of the Protein−Ligand Complex. Recombinant human FKBP12 purified from *Escherichia coli* was concentrated to 19 mg/mL in H_2O. The ligand sample was dissolved in methanol, mixed in a 1.5:1 molar ratio with FKBP12 in a vial, lightly vortexed, and then allowed to stand overnight at room temperature. For crystallization the hanging drop method was used. The hanging drops were made by mixing 5 μL of protein−drug complex with 5 μL of reservoir solution. The reservoir solution consisted of 100 mM Na/K phosphate buffer at pH 7.7 and 35% saturated ammonium sulfate. Single crystals with dimensions up to 0.6 × 0.3 × 0.3 mm^3 appeared within a week.

Data Collection. The X-ray intensity data were collected on a FAST television area detector with an Enraf-Nonius FR571 rotating anode operating at 40 kV and 80 mA. Data were measured at room temperature in 120-s frames of width 0.2° in ω and a crystal-to-detector distance of 75 mm. The evaluation of the measured intensities was performed by the program MADNES.[21] Consult Table 1 for crystallographic details.

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Structure Determination and Refinement. The structure was solved essentially by difference fourier techniques using the coordinates of the isomorphous FKBP12−rapamycin[5a] complex crystals. All refinement calculations were performed using X-PLOR[22] and model building was done using O.[23] In the initial stages of refinement the 28-*O*-methyl and cyclohexyl groups of the ligand were not included in order to obtain an unbiased Difference Fourier electron density. Water molecules were assigned to 3σ peaks in difference maps. Only those water molecules with reasonable hydrogen bond geometry and *B*-factors lower than 65 Å^2 were accepted. Conventional positional refinement and thermal parameter refinement using X-PLOR thus yielded R = 19.2% for all unique data (6870 reflections) from 8 to 2.1 Å. A subsequent difference map contoured at 3σ clearly indicated the positions of the missing 28-*O*-methyl and cyclohexyl groups which were then built into the density, yielding after refinement the final R = 18.7%. Final refinement results are given in Table 1.

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