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Structural flexibility of hyaluronan oligomers as probed by molecular modeling*

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Abstract: In the last few years, molecular modeling studies have been published that are devoted to a better understanding of the structural flexibility of hyaluronan (HA). Further conformational investigations, however, are needed on this polysaccharide, such as the application of statistical methods to perform enhanced one-step conformational analyses of its subunits. Moreover, the adjustment of assisted model building and energy refinement (AMBER) force field could provide the appropriate computational tool to study the interactions of HA and its derivatives with proteins. The present paper reports a combined Monte Carlo (MC) and molecular dynamics (MD) approach applied to the conformational study of HA, using an adjusted version of AMBER force field and the generalized Born solvent-accessible surface area (GB/SA) continuum solvation model. The MC approach turned out to be extremely effective to outline a conformational survey of the disaccharides constituting HA. Complete sets of conformations of the monomers were provided for the first time, some of which had never been predicted. MD technique, integrating the MC results, correctly reproduced the unusual stiffness of HA and predicted the existence of a minor skew-boat conformation of the β-D-glucuronic moiety. The computational approach, as a whole, improved the comprehension of the dynamic behavior of HA and offered a clear causal explanation of the relative dynamics of the glycosidic linkages.

INTRODUCTION

Hyaluronan (HA) is a member of the glycosaminoglycan family of mammalian extracellular matrix polysaccharides possessing many biological functions [1,2]. Its chemical structure is made up of repeating disaccharide units of β-D-glucuronic acid (GlcA) and 2-acetamido-2-deoxy-β-D-glucose (GlcNAc), linked by alternating β(1 → 3) and β(1 → 4) glycosidic linkages (see Fig. 1). The physical properties and functions of HA are based on its ability to form viscoelastic aqueous solutions [2].

Extensive investigations on polycrystalline samples and well-oriented fibers have shown that HA constitutes, in the solid state, helical conformations that are polymorphic [2]. The ordered conformation of HA in the solid state has raised the question about the conformation adopted by this carbohydrate in solution. Hydrodynamic measurements, characterizing the HA polymer in solution as a stiffened worm-like coil [3], have been for long interpreted as due to the presence of an extended array of intramolecular H-bonding interactions between adjacent saccharides [1]. This rather static microscopic model has

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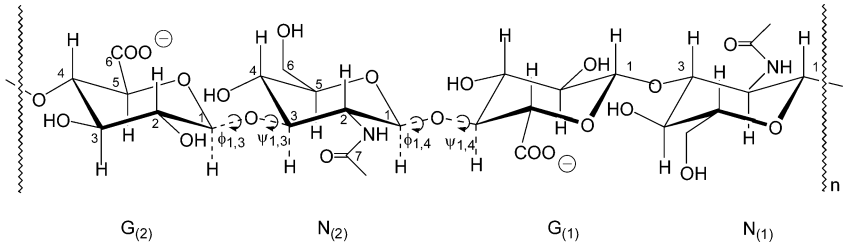

Fig. 1 Schematic drawing of the tetrasaccharide GlcA-β1,3-GlcNAc-β1,4-GlcA-β1,3-GlcNAc (G(2)-N(2)-G(1)-N(1)), considered as the repeating fragment of HA. Main atomic positions and relevant torsion angles at the glycosidic linkages (dashed bonds), are labelled.

been recently invoked to propose supramolecular organization in high-molecular-mass HA solutions [4].

The recent identification and structural characterization of numerous HA-binding proteins [5] and the modification of HA to obtain heparin-like molecules inhibiting blood coagulation [6], however, reawakened fresh interest in studying the structural properties and the associated physiological and biological roles of HA. The results of accurate molecular dynamics (MD) studies of HA oligomers [7], together with the observation that HA can originate a considerable variety of binding modes, when interacting with proteins, have suggested an alternative dynamic model of this compound in aqueous solution, as the emergent consequence of strong interactions of HA with the solvent molecules [8].

Several computational studies have been published, devoted to modeling the conformational behavior of HA. In particular, Perez et al. [9] performed the conformational study in the gas phase ($\varepsilon = 80$) of the disaccharide subunits of HA, through the computation of adiabatic maps for the glycosidic torsion angles ϕ and ψ. Differently, Almond et al. studied the secondary structure of HA through extensive MD simulations in aqueous solution of tetrasaccharide and decasaccharide subunits [7,10]. While these works have shed further light on the peculiar properties of HA in solution and in the solid state, further theoretical investigations are needed on the three-dimensional structure of HA.

The application of Monte Carlo (MC) methods to perform enhanced searches of the potential energy surface of HA subunits can be considered a primary purpose. Identifying all the important conformations in carbohydrates can be difficult, in fact, when the conformers are found by combining grid searches of few torsion angles for various starting structures [9], or when the sole MD approach is used to explore the conformational space [10]. A detailed conformational study, on the other hand, could help to find a clear causal explanation to support the dynamic model recently proposed as the result of MD studies of HA [7]. Furthermore, the use of MacroModel, a very reliable and effective software for molecular modeling, might set a proper computational tool for the study of protein–saccharide interactions. The MacroModel generalized Born solvent-accessible surface area (GB/SA) implicit solvation treatment [11], in a particular manner, reported to slow the calculations only by a factor of approximately three relative to the gas phase, would allow both the extension of the periods of MD simulations in water up to several nanoseconds and the study of bigger oligomers of HA.

Pushed by our interest of many years in the modeling of the interactions between proteins and their ligands [12], the conformational analysis of HA acid appeared the proper starting point to face future theoretical investigations on the interaction of modified hyaluronic acids with plasma proteins, aimed to assess the heparin-like behavior of these polysaccharides [13].

In the present paper, the first one-step conformational analysis of the whole monomers GlcA-β1,3-GlcNAc (G-N) and GlcNAc-β1,4-GlcA (N-G), and the exploration of the average conformation populated in solution of the dimer GlcA-β1,3-GlcNAc-β1,4-GlcA-β1,3-GlcNAc (two monomers G-N, β1,4 linked each other, $G_{(2)}$-$N_{(2)}$-$G_{(1)}$-$N_{(1)}$ hereafter) are reported. The searches have been performed using the powerful Monte Carlo multiple minimum (MCMM) searching routine [14], the MD tech-

nique, both implemented in MacroModel/BatchMin [15], change to MacroModel-AMBER force field [16] (with Homans parameters for pyranoses) [17].

METHODS

All the computational work was carried out on an SGI-Octane workstation. Molecular mechanics and dynamics calculations were performed using the MacroModel-AMBER force field as implemented in MacroModel 5.5. One torsional parameter, describing the rotation around the bond connecting C5 to COO^- in the β-D-glucuronic moiety, was added [18] with respect to the MacroModel implementation of AMBER in order to fit both the MacroModel-MM2 torsional profile of the same bond and the experimental values found in X-ray structures [19] of HA*. Accordingly, the line of the MacroModel-AMBER file corresponding to the torsion O_2=C_2(O_3)–CT–O_3 (V_1 = –0.15 kcal/mol and V_2 = 0.65 kcal/mol) replaced the original one (V_1 = 0.0 kcal/mol, V_2 = 0.0 kcal/mol, and V_3 = 0.0 kcal/mol) corresponding to entire control of the rotation by nonbonded interactions.

The extended nonbonded cutoff protocol of MacroModel was applied. The calculations were performed with two different treatments of the solvent effects: a dielectric constant of 3.0 was used in the studies performed in the gas phase, to approximate electrostatic screening from the surrounding medium [21]. The GB/SA continuum solvation method of BatchMin, differently, was used when the effects of water were directly simulated [11].

As usually done [2], the overall conformation of HA was defined by the values of the torsion angles φ and ψ at the glycosidic linkages (see Fig. 1). Furthermore, as it is widely accepted that both GlcA and GlcNAc pyranose sugars exist in the $^4C_1$ conformation in solution [2], additional conformations of the two rings were not directly searched.

Conformational analyses were performed using the MCMM procedure [14]. The FILTER option in the MacroModel framework was used to analyze the output files and collect families of conformers showing similar values of φ and ψ angles. The glycosidic linkage torsions φ and ψ, all the five secondary hydroxy group side chains, the two torsions of the hydroxymethyl group and the C–N torsion of the GlcNAc moiety, were allowed to vary in the MCMM searches on the disaccharides subunits, for a total of 10 explicit variables while maintaining the pyranose rings rigid in their $^4C_1$ chair conformation. The number of torsion angles allowed us to vary simultaneously during each MC step ranged from two to nine. A total of 10 000 search steps were performed, although after 7000 steps almost all the new minima generated were duplicates of previously found conformations. Energy minimizations were performed using the Polak–Ribiere conjugate gradient (PRCG) procedure and were terminated when the energy gradient root mean square (rms) fell below 0.01 kJ/Å mol. To eliminate duplicate conformations, a comparison was performed on the heavy atoms and hydroxyl hydrogens, selecting 0.25 Å as the maximum allowable separation between couples of corresponding atoms after superimposition. All the conformers were saved that differed from the global minimum-energy conformation by no more than 50.3 kJ/mol. All the output structures were subsequently methylated on the two OH groups corresponding to the glycosidic linkages and reminimized to leave out end-effects and get a second set of conformers comparable with those obtained in a previous conformational study of HA [9]. Only geometries differing from the global minimum-energy conformation by no more than 20.9 kJ/mol were retained.

MD simulations (in detail stochastic dynamics simulations with generation of the canonical ensemble) were performed at different temperatures (400 and 300 K) without including mobile counterions [7,10]. The starting geometries for the MD runs were systematically built connecting global minima of the monomer G-N and setting the β1,4 glycosidic linkage at its most privileged orientation. Coupling between the temperature bath and the molecules was updated every 0.2 ps. The equilibration

*The need of proper torsional parameters for the carboxy group at C6 in glucuronic acid monomers has been already noted by Altona et al., who claimed such a never-before published parametrization for AMBER force field [20].

period and the total simulation time were respectively 50 ps and 10 ns for every run. Overall molecule rotation and translation was controlled through the removal of these motions every 1 ps, while a time step of 1 fs was used [22]. During the trajectories, structures (frames) were sampled at every 1 ps throughout the time course.

RESULTS AND DISCUSSION

The currently accepted microscopic model for HA in solution is a two-fold structure stabilized by intramolecular H-bonds between neighboring sugar residues [4]. The global minimum in the gas phase of the dimer $G_{(2)}$-$N_{(2)}$-$G_{(1)}$-$N_{(1)}$ of HA, located in this study as the result of a MCMM search using an enhanced dielectric constant $\varepsilon = 3$*, and matching this structure, is shown in Fig. 2.

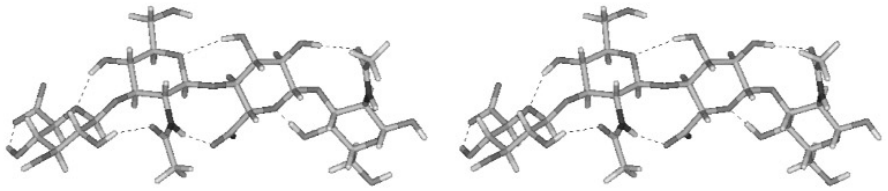

Fig. 2 Lowest energy geometry of the tetrasaccharide $G_{(2)}$-$N_{(2)}$-$G_{(1)}$-$N_{(1)}$ (stereo view) in the gas phase. Hydrogen bonds are depicted as dotted lines.

MCMM searches were performed on G-N and N-G, whose results are summarized in Tables 1 and 2, respectively.

Table 1 Energy minima of the potential energy surface of the disaccharide G-N, calculated in the gas phase with enhanced dielectric constant (upper part, within a range of 20.9 kJ/mol over the global minimum) and in GB/SA water (lower part, within a range of 12.6 kJ/mol over the global minimum).

Family of conformers	ΔE (kJ/mol)	φ_{1-3} (°)	ψ_{1-3} (°)	H-bond
1	0.0	50	–8	O_5(G) – HO_4(N)[a]
				O_2H(G) – O_7(N)[a]
2	2.9	171	8	O_2(G) – HO_4(N)
				O_5(G) – HN(N)
3[b]	8.4	44	170	O_6(G) – HN(N)
4	13.0	77	60	//
5	20.1	–28	–16	O_2(G) – HN(N)
1	0.0	48	–6	O_5(G) – HO_4(N)
				O_2H(G) – O_7(N)
2	9.2	170	6	O_5(G) – HN(N)

[a]See Fig. 2.
[b]The 2-acetamido group shows a *cis* orientation.

Five (φ,ψ) conformational families of methylated conformers of G-N were classified in the gas phase within a range of 20.9 kJ/mol from the global minimum, whose minima are named 1–5 in the

*An MCMM search in the gas phase was performed at the β1,4 glycosidic linkage of the dimer to select its most privileged orientations.

upper part of Table 1. The geometry of minimum 1 appeared to correspond to the average conformation, mainly populated at room temperature, already secured in previous studies on HA [23] (matching the structure shown in Fig. 2). A second family (reported as 2 in Table 1) was located at a ϕ value of around 170°, and its minimum was found to possess a steric energy 2.9 kJ/mol higher than the global minimum. According to the exoanomeric effect, $\phi = 171°$ roughly corresponds to the less favored minimum of the C1–O torsional profile, but, in the case of the disaccharide G-N, two intramolecular H-bonding interactions between the adjacent sugars seemed to stabilize conformation 2. Finally, families 3–5 were found to possess much higher steric energy than 1. Conformation 3 showed the acetamido group in a *cis* orientation. Conformation 4, consistent with the exoanomeric effect, was not stabilized by intramolecular H-bonding interactions, while conformation 5 was strongly disfavored by the exoanomeric effect and showed high steric energy despite the presence of one H-bonding interaction between the two adjacent sugars.

The results of the conformational analysis of G-N were found to be strongly affected by GB/SA implicit water treatment. In this case, the chosen dielectric constant value ($\varepsilon = 1$) allowed to consider the steric energy differences as reliable values from which Boltzmann populations were evaluated [24], and consequently, only conformations found to be populated at room temperature ($\Delta E = 12.6$ kJ/mol) were considered and reported in Table 1 (lower part). While the major structural features of minima 1 and 2 were almost unchanged, a sharp increase of the steric energy of all the conformations was observed with respect to the global minimum so that only conformations 1 (98 %) and 2 (2 %) were accessible at room temperature.

Table 2 Energy minima of the potential energy surface of the disaccharide N-G, calculated in the gas phase with enhanced dielectric constant (upper part, within a range of 20.9 kJ/mol over the global minimum) and in GB/SA water (lower part, within a range of 12.6 kJ/mol over the global minimum).

Family of conformers	ΔE (kJ/mol)	φ_{1-3} (°)	ψ_{1-3} (°)	H-bond (dist. Å)
6	0.0	39	19	NH(N) – O_2C(G)[a]
				O_5(N) – HO_3(G)[a]
7	11.7	29	172	O_7H(N) – O_2C(G)
8	13.8	171	6	NH(N) – O_3(G)
				O_7H(N) – O_2C(G)
9	14.2	80	59	O_7H(N) – O_2C(G)
10[b]	0.0	50	–10	O_5(N) – HO_3(G)
6	0.8	32	26	NH(N) – O_2C(G)
11[b]	5.0	15	–50	//
8	5.9	171	3	NH(N) – O_3(G)
				O_7H(N) – O_2C(G)
7	7.1	34	171	O_7H(N) – O_2C(G)
9	7.1	75	60	O_7H(N) – O_2C(G)
12	12.2	–52	–74	NH(N) – O_3(G)

[a]See Fig. 2.
[b]The 2-acetamido group shows a *cis* orientation.

In the case of N-G, four (φ,ψ) conformational families (named 6–9 in the upper part of Table 2) were classified in the gas phase within a 20.9 kJ/mol energy window, but only the global minimum appeared to be populated to a high degree at room temperature. Moreover, and differently from the case of G-N, conformation 6 showed φ and ψ values slightly dissimilar with respect to the values found in previous studies on HA [10,23]. The above results were completely changed when water effects were

directly simulated. Several conformational families (6–12 in the lower part of Table 2) were found to be populated at room temperature due to solvation by water and surprisingly, the new minima 10 (new global minimum, possessing the expected φ and ψ values [23]) and 11 (ΔE_{10-11} = 5.0 kJ/mol, no H-bonding interactions between saccharide units) showed the acetamido side chain *cis* oriented with respect to the H atom at C2. In this orientation, no H-bonding interactions could be formed between this side chain and the carboxy group.

HA's higher mobility of the β(1 → 4) linkage with respect to the β(1 → 3) one has been already experimentally observed [23] and theoretically predicted as "an emergent property of the combined water-polymer ensemble" [7]. According to the findings of this conformational study, such a property, stressed by the solvation effects of water, is due to peculiar structural features of HA: the formation of one of the two proposed direct H-bonds between the adjacent saccharide units [shown in Fig. 2 between sugars $N_{(2)}$ and $G_{(1)}$] is disfavored with respect to the interaction via the solvent (water bridges) for the relative positions in 3D space, of the *cis-trans* flexibile acetamido side chain at C2 of GlcNAc, and of the carboxy side chain at C5 of GlcA (only one torsional degree of freedom). The φ value of minimum 6 in the gas phase (39°), which optimizes the formation of the H-bond between the acetamido and carboxy groups, is far from the ideal exoanomeric value (60°) and is evidence of this situation.

The global minimum of G-N, not methylated [10], was then subjected to an MD study in order to calibrate the optimal thermodynamic conditions, namely temperature and treatment of the solvent effects, to perform the compulsory MD conformational study of oligomers of HA. Furthermore, the time evolution of the molecular motions of the disaccharide was expected to let the systems move between conformations populated at room temperature, crossing low-energy barriers so as to provide a dynamic picture of the structure. A first set of runs was performed at different temperatures (400 and 300 K) in the gas phase, enhancing to 3 the value of the dielectric constant, and a final 10 ns MD simulation was performed at 300 K using the GB/SA continuum solvation model for water.

In the runs at 400 K, performed to favor the crossing of conformational energy barriers and allow a wider sampling of the conformational space during relatively short simulations [25], few changes were observed involving both the glycosidic linkage and the acetamido group. At 300 K, exceptional changes involving the glycosidic linkage of G-N toward conformation 2 were observed in both the environments, while the acetamido group, bounded in the *trans* orientation in the gas phase, underwent several *trans-gauche* conformational changes in GB/SA water. The fact that the well around conformation 2 resulted rarely populated during the MD runs of G-N, in spite of its apparent accessibility in the gas phase (pointed out in the MCMM search, see Table 1), could indicate the valley around 2 is narrower than the one around 1. According to this hypothesis, conformation 2 would be much less populated than 1 due to a lower statistical weight determined by the proportionately smaller number of vibrational microstates [26].

At 400 K, a conformational change of the glucuronate ring was observed, lasting several hundreds of picoseconds, out of the $^4C_1$ chair toward a $^5S_3$ (^2S_O) skew-boat conformation (shown in Fig. 3), stabilized by a dipolar attractive interaction between the carboxylate of G moiety and the OH group at C6 of the N moiety. Interestingly, a ^2S_O skew-boat conformation has been recently reported in a NMR study in water [27], as a minor conformation of the β-D-glucuronic moiety at the nonreducing terminus of a tetrasaccharide from HA. The results of the MD simulations reported here constitute, to our knowledge, the first theoretical outcome of that experimental result.

Both the MD simulations on the disaccharide G-N performed at 400 K in the gas phase (ε = 3) and at 300 K in GB/SA water gave results in reasonable agreement with experimental data and previous MD studies [10] and allowed the reproduction, for the first time, of a whole set of NMR vicinal ^3J-coupling constants*. The simulation at 300 K in GB/SA water, particularly, allowed to calculate a (φ,ψ) population distribution of G-N comparable with that obtained by Almond et al. in their computer-

*Results not shown.

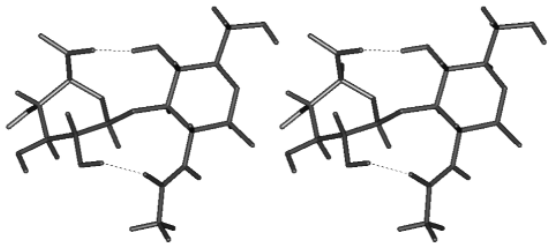

Fig. 3 Stereo view of an energy minimized geometry of the monomer G-N, detected in the MD run performed at 400 K, in which the glucuronate ring is observed in the uncommon $^{2}S_{O}$ skew-boat conformation. Hydrogen bonds are depicted as dotted lines.

demanding MD study on the disaccharides of HA [10b], performed in aqueous solution including 1000 explicit water molecules. MD simulations on the dimer $G_{(2)}$-$N_{(2)}$-$G_{(1)}$-$N_{(1)}$, performed applying the same protocol, confirmed the above picture. Two main H-bonds were observed at 400 K across the $G_{(1)}$-$N_{(1)}$ $\beta(1 \rightarrow 3)$ linkage (see Fig. 2), the interaction involving the ring oxygen of $G_{(1)}$ and the O_4H of $N_{(1)}$ (found in the 50 % of frames) and the one between the acetamido group (CO) of $N_{(1)}$ and the O_2H of $G_{(1)}$ (11 % of the frames), whose low persistence correctly reproduced extra flexibility at the reducing terminus of the dimer [10]. The same H-bonding interactions were found respectively in the 100 % and 79 % of frames at the $\beta(1 \rightarrow 3)$ junction between $G_{(2)}$ and $N_{(2)}$, while the mobility about the internal $\beta(1 \rightarrow 4)$ glycosidic linkage was higher and limited by the formation of two H-bonding interactions, one involving the ring oxygen of $N_{(2)}$ and the O_3H of $G_{(1)}$ (found in the 47 % of the frames), the other involving the acetamido NH of $N_{(2)}$ and the carboxylate of $G_{(1)}$ (found in the 45 % of the frames). Notably, these last values mirrored the results of MCMM searches on the monomers and confirmed the conformational flexibility of the acetamido side chain.

In conclusion, the MC searches found several conformational families of the monomers G-N and N-G, some of which were never detected before, providing a complete set of conformations and establishing the higher efficiency of this methodology with respect to multistep conformational search protocols. Thanks to this approach, the reported higher mobility of the $\beta(1 \rightarrow 4)$ linkage with respect to the $\beta(1 \rightarrow 3)$ linkage was grounded both to structural features of HA and solvation effects by water. The MD approach provided a satisfactory description and clear interpretation of the molecular motions of HA and confirmed the recently proposed dynamic model of this molecule. Notably, in MD simulations at 400 K, a skew-boat conformation of the glucuronate ring, already reported for the *L*-iduronate residue [2] and previously detected in a NMR study of a tetrasaccharide of HA, was theoretically predicted for the first time. Our computational approach (not CPU demanding), proved to be rapid and reliable, therefore suitable for further deeper investigations on tertiary structures of HA in aqueous solution [4] or on the interactions of HA (eventually modified) with proteins.

REFERENCES

1. L. Lapčik Jr., L. Lapčik, S. De Smedt, J. Demeester, P. Chabreček. *Chem. Rev.* **98**, 2663 (1998).
2. V. S. R. Rao, P. K. Qasba, P. V. Balaji, R. Chandrasekaran. *Conformation of Carbohydrates*, pp. 258–263 and references cited therein, Harwood Academic, Amsterdam (1998).
3. R. L. Cleland. *Biopolymers* **23**, 647 (1984).
4. J. E. Scott and F. Heatley. *Proc. Natl. Acad. Sci. USA* **96**, 4850 (1999).
5. A. J. Day and G. D. Prestwich. *J. Biol. Chem.* **277**, 4585 (2002).
6. R. Barbucci, A. Magnani, R. Rappuoli, S. Lamponi, M. Consumi. *J. Inorg. Biochem.* **79**, 119 (2000).
7. A. Almond, A. Brass, J. K. Sheehan. *J. Phys. Chem. B* **104**, 5634 (2000).

8. A. J. Day and K. J. Sheehan. *Curr. Opin. Struct. Biol.* **11**, 617 (2001).
9. K. Haxaire, I. Braccini, M. Milas, M. Rinaudo, S. Pérez. *Glycobiology* **10**, 587 (2000).
10. (a) A. Almond, A. Brass, J. K. Sheehan. *Glycobiology* **8**, 973 (1998); (b) A. Almond, A. Brass, J. K. Sheehan. *Glycobiology* **7**, 597 (1997).
11. W. C. Still, A. Tempzyk, R. Hawley, T. F. Hendrickson. *J. Am. Chem. Soc.* **112**, 6127 (1990).
12. A. Tafi, A. van Almsick, F. Corelli, M. Crusco, K. E. Laumen, M. P. Schneider, M. Botta. *J. Org. Chem.* **65**, 3659 (2000).
13. F. Manetti, F. Corelli, M. Botta. *Curr. Pharm. Des.* **6**, 1897 (2000).
14. G. Chang, W. C. Guida, W. C. Still. *J. Am. Chem. Soc.* **111**, 4379 (1989).
15. F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, W. C. Still. *J. Comput. Chem.* **11**, 440 (1990).
16. D. Q. Mc Donald and W. C. Still. *Tetrahedron Lett.* **33**, 7743 (1992).
17. S. W. Homans. *Biochemistry* **29**, 9110 (1990).
18. J. A. Tucker, K. N. Houck, B. N. Trost. *J. Am. Chem. Soc.* **112**, 5465 (1990).
19. J. M. Guss, D. W. L. Hukins, P. J. C. Smith, W. T. Winter, S. Arnott, R. Moorhouse, D. A. Rees. *J. Mol. Biol.* **95**, 359 (1975).
20. C. J. M. Huige and C. Altona. *J. Comput. Chem.* **16**, 56 (1995).
21. W. von der Lieth, T. Kozár, W. E. Hull, *J. Mol. Struct. (Theochem)* **395–396**, 225 (1997).
22. B. Botta, M. Botta, A. Filippi, A. Tafi, G. Delle Monache, M. Speranza. *J. Am. Chem. Soc.* **124**, 7658 (2002).
23. S. M. A. Holmbeck, P. A. Petillo, L. E. Lerner. *Biochemistry* **33**, 14246 (1994).
24. A. Gambacorta, D. Tofani, A. Tafi, M. Elmi Farah. *Tetrahedron* **57**, 5435 (2001).
25. Y. Sun and P. A. Kollman. *J. Comput. Chem.* **13**, 33 (1992) and references cited therein.
26. A. Leach. In *Reviews in Computational Chemistry* **2**, K. B. Lipkowitz and D. B. Boyd (Eds.), pp. 1–55, VCH, New York (1991).
27. P. Livant, L. Rodén, N. R. Krishna. *Carbohydr. Res.* **237**, 271 (1992).