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2D NMR and conformational analysis of a prototype anti-tumour steroidal ester

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Abstract

The synthetic 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*-*N*-bis(2-chloroethyl)aminophenylacetate (SOT-19, **I**) was found to be a very potent anti-leukaemic agent candidate. Its high biological activity and low toxicity rationalize the study of its conformational properties. It can also serve as a prototype and therefore as a template for a series of congener compounds possessing a variety of toxicity and anti-leukaemic activity in subsequent 3D-QSAR studies. Its low energy conformers were identified through a combination of conformational search methods and 2D NOESY NMR spectroscopy. The low energy conformers were mainly compact, with the alkylating aromatic group orienting either to the α- or β-surface of the steroidal plane. The preference in the orientation of the alkyl chain may be steroid dependent and related to the mechanism by which they produce their anti-leukaemic action. This hypothesis is supported by the fact that small chemical modifications of the conformation on the steroidal skeleton produce significant alterations on the anti-leukemic activity.

Keywords: Anti-tumour steroidal esters; Nitrogen mustards; 2D NOESY NMR; Conformational analysis; Molecular modeling; Chlorambucil

1. Introduction

The major defects of current anti-cancer therapies are the serious side effects and/or the induced resistance to the drugs or the procedures that are being used. Nitrogen mustards are the earliest and most extensively studied DNA-interstrand cross-linking agents, which constitute a category of anti-cancer drugs tested and proven through the years successful in myelogenic leukaemia, Hodgkin disease, lung, testicle, ovarian and breast cancer, as well as in several lymphomas [1]. Despite their long history, only a few members of these anti-cancer compounds, such as melphalan and chlorambucil, are used in clinical cancer chemotherapy today [2,3]. Due to their high intrinsic chemical activity, they can bind covalently to the nucleophilic sites of several biomolecules. Their effective alkylating capacity regarding DNA however, is diminished for two reasons; firstly, they show relatively low affinity and only slight selectivity for longer DNA sequences and secondly, they get rapidly hydrolyzed before reaching the DNA-target [4].

The chemical conjugation of nitrogen mustards to carrier-molecules is one of the strategies, which researchers apply in order to reduce the toxicity of these alkylating agents and increase selectivity and effectiveness towards alkylation of DNA [5,6]. Estramustine and prednimustine, nitrogen mustard carbamates of steroids with clinical use [7], are typical models of conjugation of an alkylating agent to steroids. From our laboratory's previous projects with a series of aromatic aniline mustards linked to simple or D-ring modified steroidal molecules, there is strong evidence for a significant enhancement of the anti-leukaemic activity and a re-

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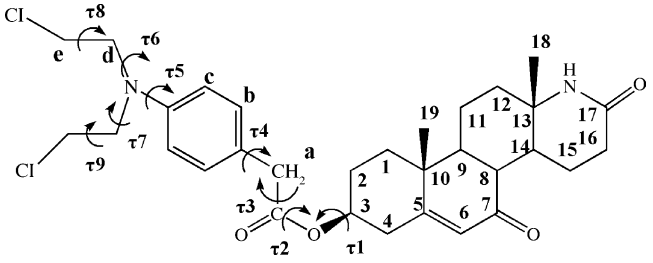


Fig. 1. 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*,*N*-bis(2-chloro-ethyl) aminophenylacetate (**I**) comprises an alkylating agent and a modified steroid with an expanded lactamic D-ring. Torsions τ1–3 were used as variables during conformational search with grid scan, while all 9 torsions were included in random sampling.

duction of the toxicity of these compounds compared with the nitrogen mustards themselves [8–10]. Moreover, an early study with four 7-keto steroidal esters of several nitrogen mustards [11] produced promising results regarding the anti-leukaemic effectiveness of the introduction of a 7-keto group in the steroidal skeleton of the final esteric derivatives. However, the amelioration of the pharmacological profile in all cases is so great that it seems reasonable for one to investigate whether there is much more than the pharmacokinetics of the alkylating agents that is affected by this chemical combination.

The above reasons rationalize the study of the conformation of one characteristic representative of this category of compounds, such as 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*,*N*-bis(2-chloroethyl)aminophenylacetate (SOT-19, **I**) (Fig. 1). This derivative contains as an alkylating agent chlorambucil's active metabolite (*p*-*N*,*N*-bis(2-chloroethyl)aminophenylacetic acid) [12], while the steroidal skeleton (3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione) comprises successful modifications on the androstane skeleton, such as the lactamic D-ring and the allylic 7-keto groups. Since it possesses high anti-leukaemic activity and moderate toxicity in vitro and in vivo [13], it can be considered as a prototype and therefore a template for a series of analogous derivatives, which are to be analyzed through QSAR studies. The conformational analysis was considered necessary in order to explore its low energy conformers and to find any specific information about its putative bioactive conformation, which will serve as a template for building a proper database of its derivatives in a forthcoming 3D-QSAR analysis. Structure elucidation and conformational analysis was carried out with the use of 1D and 2D NMR spectroscopy in combination with computational analysis.

Experiments were performed in $CDCl_3$ ($\varepsilon = 1$), a convenient organic solvent since its signal on the ^{1}H NMR spectrum does not overlap with the signals of **I**. The choice of $CDCl_3$ may not necessarily best simulate the biological environment. However, our under progress CoMFA and CoMSIA QSAR studies will establish the validity of the selection of this environment. If a good correlation between conformation and bioactivity is not established, other environments (micelles, bicelles, vesicles, amphiphathic or polar solvents), which simulate more closely the biological environment may have to be tested.

2. Experimental

2.1. Nuclear magnetic resonance experiments

The purity of **I** was checked by HPLC (waters, MILLIPORE®). Its purity and structure identity were confirmed by comparison of its 1D ^{1}H NMR spectrum with the corresponding spectrum of its steroidal precursor. $CDCl_3$ (99.96%) and ultra precision NMR tubes Wilmad 535–5 mm (SPINTEC ROTOTEC) were used for the NMR experiments. High-resolution spectra were obtained with the use of the Varian INOVA 600 MHz and Bruker AC 300 instruments at 298 K. All data were collected with the pulse sequences and phase-cycling routines provided in the Bruker and Varian libraries of pulse programs. Data processing, including sine-bell apodization, Fourier transformation, phasing, symmetrization and plotting, were performed with the use of the Varian and Bruker software packages. The optimum mixing time for the NOESY spectrum of **I** was found to be 150 ms.

2.2. Molecular modeling and conformational search

The structure of **I** was built with the use of QUANTA 97 (CHARMm) on an S/G O2 platform and was minimized with the application of the Adopted Basis Newton Raphson (ABNR) minimization algorithm. The dielectric constant was set to 1 in order to simulate that of the solvent used in NMR experiments. The minimized structure was subjected to both a systematic (grid scan) and a stochastic (random sampling) search procedures. Both methods proved to be efficient in predicting the lowest energy conformations and provided similar results. The output conformation files were repeatedly subjected to minimization with the use of the ABNR, Powell and Steepest Descents algorithms. The torsion angles used as variables during conformational search are shown in Fig. 1. Initially, grid scan was performed to explore the conformational space determined by torsion angles τ1, τ2 and τ3 (torsion step: 30°) that seem to define the orientation of the alkylating part in relation to the steroidal ring system. The analysis of the minimized conformations was facilitated by a clustering procedure in which the use of a torsion threshold criterion resulted in the creation of 16 clusters. The lowest energy conformer from each cluster was selected and the 16 conformations were compared in relation to their accordance with the experimental conformational data, that is, the observed NOEs. Afterwards, random sampling was performed and all 9 torsions (torsion window: 180°) were used. The output conformation files were subjected to minimization with the use of the ABNR and Powell algorithms, both with and without atom distance constraints.

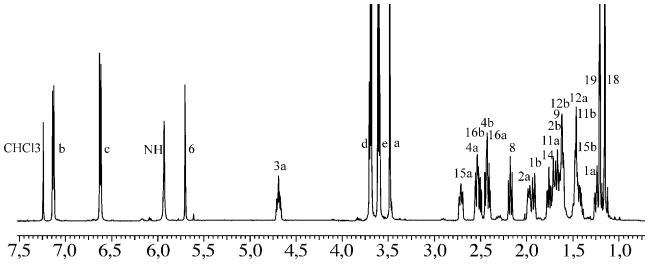


Fig. 2. The ^{1}H NMR spectrum of **I**, fully assigned.

3. Results

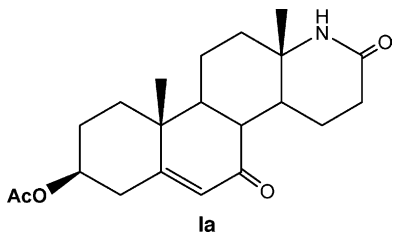
3.1. Structure elucidation

The ^{1}H NMR spectrum of **I** was fully assigned (Fig. 2) with the use of a combination of 1D NMR and 2D homonuclear and heteronuclear NMR spectroscopy. The previous full assignment of the corresponding NMR spectra of the steroidal precursor of **I** (Fig. 3) aided considerably in the structure elucidation [14]. The ^{1}H NMR spectrum of **I** consists of two regions, one that accounts for the protons of the steroidal ring system and another for the protons of the alkylating part. The protons of the two systems are not coupled via bond since the –O–CO– group separates them. This fact simplifies the assignment, and information from the spectra of the steroidal part can be safely applied to the spectra of **I**. The assignment of the protons of the alkylating part on the ^{1}H NMR spectrum was straightforward.

Table 1 shows the observed chemical shifts for **I** and its steroidal precursor **Ia**. As it can be seen, the chemical shifts of the steroidal ring in the two molecules are very similar. The direct comparison of the chemical shifts limits the scope of 2D NMR spectroscopy only to a confirmatory role.

Once fully assigned the ^{1}H NMR spectrum of **I**, the identification of critical NOEs was pursued on the corresponding NOESY spectrum (Fig. 4). Critical NOEs are the ones that determine the orientation of the alkylating segment relative to the steroidal segment.

The effort to quantify the NOE signals obtained on the 300 and 600 MHz spectrometers was not successful due to the prohibiting overlapping of the critical NOEs. To overcome this problem a novel strategy, depicted in the flow chart of Fig. 5, was applied, which makes the use of volume calculation unnecessary.



Ia

Fig. 3. The full assignment of the ^{1}H NMR spectrum of **I** was based on the previous assignment of the HSQC, HMBC and DQF-COSY spectra of its steroidal precursor **Ia** (3β-acetoxy-17α-aza-D-homo-5-androsten-7,17-dione).

Table 1
Chemical shifts for **I** and its steroidal precursor **Ia**

I	**Ia**
1a: 1.23	1a: 1.23
1b: 1.92	1b: 1.93
2a: 1.96	2a: 1.97
2b: 1.66	2b: 1.67
3a: 4.68	3a: 4.68
4a: 2.53	4a: 2.54
4b: 2.42	4b: 2.42
6: 5.69	6: 5.72
8: 2.17	8: 2.18
9: 1.66	9: 1.66
11a: 1.70	11a: 1.70
11b: 1.46	11b: 1.46
12a: 1.47	12a: 1.47
12b: 1.62	12b: 1.62
14: 1.75	14: 1.75
15a: 2.71	15a: 2.71
15b: 1.42	15b: 1.42
16a: 2.41	16a: 2.41
16b: 2.51	16b: 2.51
18: 1.15	18: 1.15
19: 1.21	19: 1.21
N–H: 5.92	N–H: 6.19
–	CH_3–CO: 2.02
a: 3.47	–
b: 7.12	–
c: 6.61	–
d: 3.69	–
e: 3.59	–

In the conformational analysis of **I**, the theoretical calculations generated low energy conformers consistent with the NOE data. In order to confirm the results, NOEs were used in a qualitative way to generate low energy conformers. The agreement between theoretical and experimental data allowed us to proceed without calculation of the NOE volumes.

3.2. Conformational analysis

Firstly, as already mentioned, grid scan was performed with torsions τ1–3 as variables, and a torsion step of 30°. The output conformation file was repeatedly subjected to energy minimization with the use of the Newton Raphson and Steepest Descents minimization algorithms.

The final file was subjected to clustering by torsion and 16 clusters were created. From the superposition of the 16 lowest energy representatives of each cluster (Fig. 6) it can be clearly seen that there are three possibilities for the orientation of the alkylating part of the molecule relative to the steroidal part.

The alkylating part either orientates to the α-surface or to the β-surface, or is kept "open" and away from the steroidal part. Generally the lowest energy conformers are those that adopt the β-orientation, shown superimposed in Fig. 7.

Critical distances between atoms in each conformation were calculated and compared to the most important observed NOEs (b-19, c-19, d-19, e-19, c-4b, d-4b, e-4b and e-6, de-

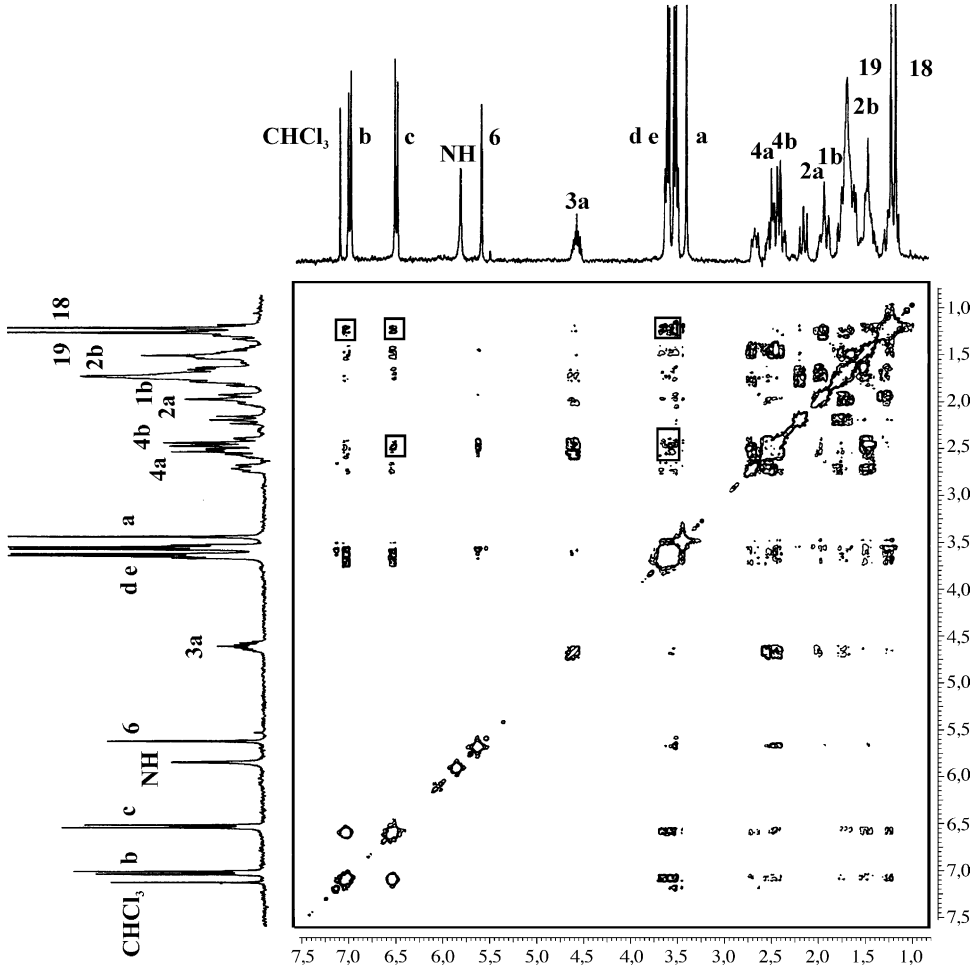


Fig. 4. The characteristic NOEs among the protons of the steroidal and the alkylating parts are shown on the NOESY spectrum of **I**.

scribed in the second row of Table 2). It was assumed that calculated distances of $\leq 5 \pm 0.20$ Å can satisfy the observed NOEs. Under this assumption, conformer ***1*** satisfies all observed NOEs, while conformers ***3*** and ***4*** violate only the c-19 NOE. Differences among these conformers are small and mainly concern the orientation of the one of the $ClCH_2CH_2$-chains, a quite flexible part of the molecule. Thus, it seems reasonable for one to select the lowest energy conformer as the most plausible. Conformer ***9*** does not satisfy an important NOE (e-6), its conformation differs significantly from that of conformer's ***1*** and its relative energy is 5.31 kcal/mol greater.

However, in a biological environment this amount of energy can be overcome and such conformational changes can be afforded. Thus, all above-mentioned conformers can still be considered as putative bioactive conformations

In order to confirm the results and investigate the influence of the value of torsions τ4–9 on the conformation and relative energy of the molecule, as it was not computationally possible with grid scan in the software used, random sampling was performed. All 9 torsions were used and the torsion window was set to 180°. The output conformation file was processed both without any constraints, and with the constraints resulting from the observed NOEs (Table 2), for comparison. Conformer ***2**** (energy: 15.89 kcal/mol), identical to conformer ***1***, is the lowest energy conformer resulting from processing without any constraints, while ***3**** (energy: 16.77 kcal/mol) is the lowest energy conformer resulting from processing the random sampling output file with atom distance constraints applied. Neither run produced any lower energy conformation than conformer ***1***, though conformer ***3**** fulfills all the NOE criteria. A comparison of the critical distances of conformers ***1*** and ***3**** is shown in Table 3.

Conformers ***1*** and ***3**** are shown in Fig. 8, in which it is obvious that the steroidal part adopts similar conformations, and torsion angles τ1–3 have the same values in both cases. The two conformers differ slightly and only in the orientation of the aromatic ring and the $Cl–CH_2CH_2$, which is close to the steroidal part; these differences cost less than 1 kcal/mol. The above results indicate that the energetically favored conformation of 3β-hydroxy-17α-aza-D-homo-5-androsten-7,17-dione-*p*-*N*-*N*-bis(2-chloro-ethyl) amino phenylacetate (**I**), resulting from theoretical computation only (***1***), is very close to the conformation resulting from a combination of theoretical computation and experimental data (***3****).

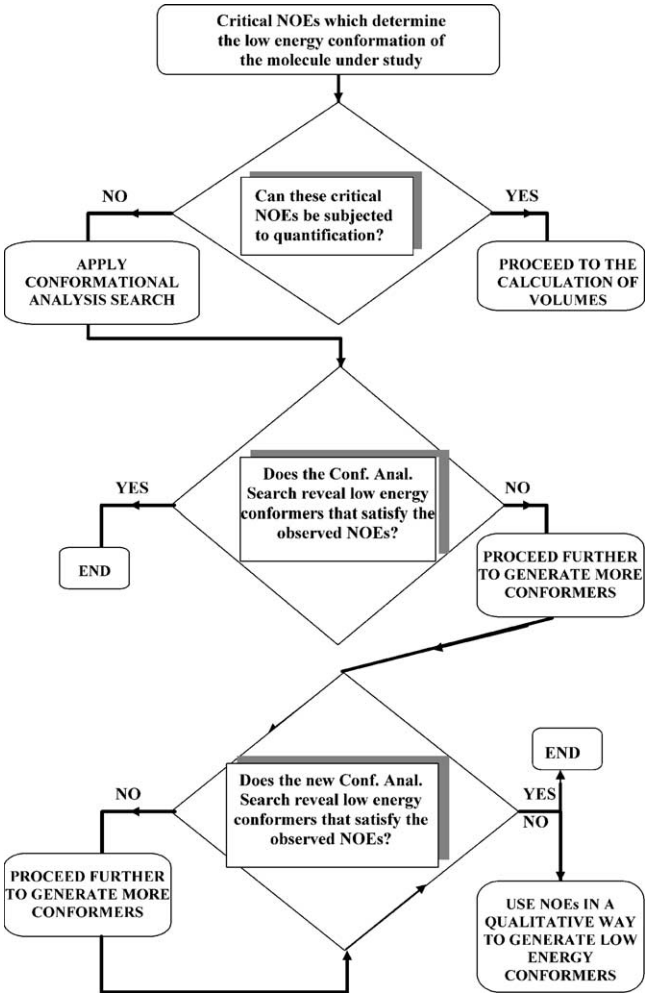


Fig. 5. In the case of problematic NOE volume calculation, a procedure of several conformational search iterations is proposed. Eventually, if the obtained conformers do not satisfy the NOEs, the latter can be used in a qualitative way to generate low energy conformers.

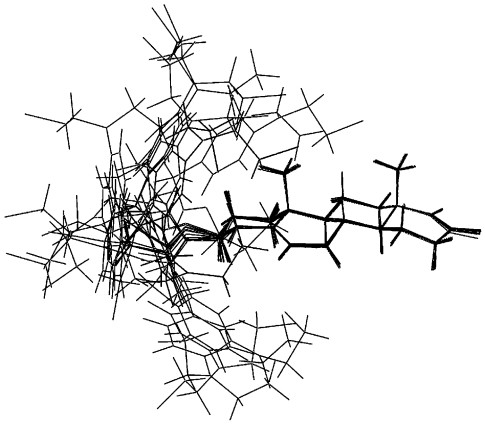


Fig. 6. The 16 lowest energy conformations of each cluster are shown in superposition.

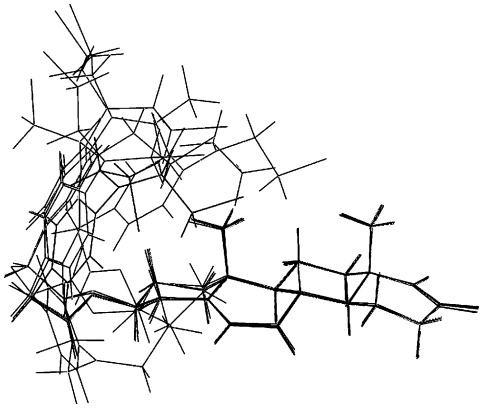


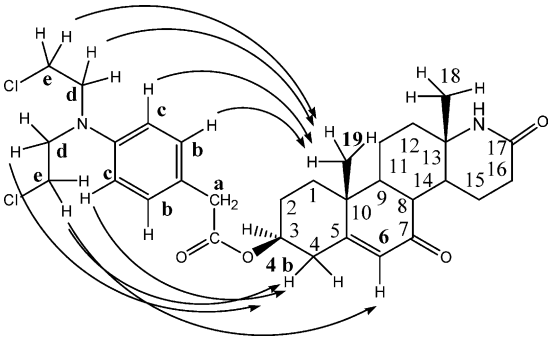
Fig. 7. Low energy conformers characterized by the adoption of a β-surface orientation of the alkylating part are shown in superposition.

4. Discussion

The lowest energy conformation of **I** in solution has been successfully identified with the combined use of experimental and theoretical data. The 1D and 2D NMR spectra of **I** and its steroidal precursor aided the structural elucidation. The phase sensitive NOESY spectrum of **I** defined the spatial connectivities of protons. Conformational analysis based on systematic search with grid scan showed that **I** can adopt different low energy conformations with the alkylating group protruding either to the α- or β-surface of the steroidal plane to form compact structures, or remaining away from the steroidal skeleton. Generally, the lowest energy conformers are the ones that adopt the β-orientation, in good agreement with the NOE data. Conformational analysis based on stochastic search with random sampling confirmed the preference for the β-orientation and the values of torsion angles τ1–3. Additionally, the minimization treatment of the random sampling output file with the application of constraints derived from the NOE data, revealed the importance of the orientation of the aromatic ring and the $Cl–CH_2CH_2$ groups. Conformer ***3**** is the lowest energy conformation that fulfils the NOE constraints (generated after the ^1H_a–^1H_b, which showed NOEs, were imposed to have a spatial distance of $\leq$5 Å). The dihedral angles of ***1*** and ***3**** are very similar, indicating that the theoretical approach and the application of a combination of theoretical calculations with experimental data produce similar results. These studies must be followed by QSAR, since the environment used in the experiments may not be the optimum and the exact mechanism of action of these compounds still remains unsolved. Early studies [13] indicate that the participation of the steroidal moiety must be more than a simple "carrier", because of the significant alterations in the anti-leukemic activity produced by slight chemical modifications which may affect the conformational properties of the steroidal skeleton.

It is stated in the literature that molecules possessing an alkyl chain, are subjected to conformational changes when

Table 2
1–16 are the corresponding lowest energy conformations from each of the 16 clusters



Conformer number (energy: kcal/mol)	Observed NOEs							
	b-19	c-19	d-19	e-19	c-4b	d-4b	e-4b	e-6
	Calculated distances							
1 (15.89)	***5.01***	***4.99***	***5.08***	***2.92***	***4.44***	***4.30***	***2.93***	***5.07***
2 (18.13)	5.30	*x*	*x*	*x*	*x*	*x*	*x*	*x*
3 (18.51)	***4.73***	***x***	***3.76***	***3.42***	***4.02***	***3.87***	***2.59***	***3.66***
4 (18.89)	***5.09***	***x***	***4.53***	***2.60***	***4.13***	***4.89***	***2.65***	***5.18***
5 (20.41)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
6 (20.53)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
7 (20.58)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
8 (21.07)	4.59	3.95	2,47	3.74	4,3	3.42	*x*	*x*
9 (21.20)	***4.89***	***4.40***	***3.31***	***2.34***	***4.50***	***4.97***	***3.22***	***x***
10 (22.26)	*x*	*x*	*x*	5.45	5.07	*x*	3.71	2.82
11 (24.35)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
12 (24.86)	*x*	*x*	*x*	*x*	4.75	*x*	*x*	2.74
14 (25.60)	3.18	4.06	4.06	2.28	*x*	*x*	*x*	*x*
14 (31.72)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
15 (33.55)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*
16 (35.73)	*x*	*x*	*x*	*x*	*x*	*x*	*x*	*x*

For each conformer the critical distances between protons corresponding to the observed NOEs were calculated. The conformations compatible with most NOE data are shown in bold and italics. Marked with *x* are distances that have values greater than 5.50 Å.

placed in different environments. For example, the alkyl chain of the psychoactive cannabinoid (−)-Δ^8tetrahydrocannabinol (Δ^8-THC) undergoes a trans:gauche isomerization, protruding towards the phenolic hydroxyl aromatic ring when accommodated in bicelles. Thus, Δ^8-THC adopts a more compact conformation when accommodated in bicelles, than in $CDCl_3$ or SDS [15]. Similar effects were observed with losartan, an AT1 antagonist [16] and β-Ala-Tyr, a dipeptide neuroactive toxin [17].

However, in the case of **I**, this compact conformation is favored even in the $CDCl_3$ solution, indicating that its putative bioactive conformation is probably unique, even in more complex media that simulate the biological environment. From this study it is apparent that the steroidal part must govern the interactions with the aromatic and alkyl moieties of **I**.

Our studies of the thermal effects of **I** in lipid bilayers shows that **I** is a strongly perturbing agent [18]. This confirms the NMR results, which show a strong interaction among the alkyl and the aromatic moieties of the alkylating part and the steroidal part. If such a strong interaction was absent, the perturbation would be expected to be weak. These experiments confirm our presupposition that strong Van der Waals interactions between hydrophobic moieties are predominant in the possible bioactive conformations of the molecule. Having established the first driving force that governs the conformation of **I**, a forthcoming QSAR study is expected to

Table 3
A comparison of conformers ***1*** and ***3**** is shown, regarding the accordance of the critical theoretical distances of each with the observed NOEs

Conformer number (energy: kcal/mol)	Observed NOEs							
	b-19	c-19	d-19	e-19	c-4b	d-4b	e-4b	e-6
1 (15.887)	5.01	4.99	5.08	2.92	4.44	4.30	2.93	5.07
3* (16.773)	3.92	3.81	3.85	4.54	3.80	4.01	3.55	4.56

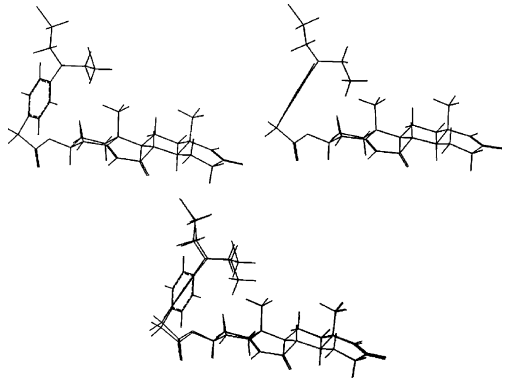


Fig. 8. Conformer **1** (left, top) is the lowest energy conformation resulting from the minimization processing of the grid scan search file. Conformer **3*** (right, top) is the corresponding lowest energy conformation resulting from the minimization processing of the random sampling search file with the application of atom distance constraints. The two conformers are also shown in superimposition (bottom).

reveal the subtle stereoelectronic factors responsible for activity.

In conclusion, the conformational analysis of **I** provided putative bioactive conformations, which will serve as templates for 3D-QSAR studies of a series of **I**'s congeners. The necessity and credibility of NMR in 3D-QSAR studies as a method for defining the bioactive conformations of flexible molecules was discussed by the authors in [19] and remains to be confirmed.

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