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^{1}H NMR Analysis of Isocyclosporin A Prepared in Organic Solvent and in Aqueous Solution

Lolita Arnoldi, Ada Manzocchi,* Fulvio Magni, Marina Del Puppo,* and Marzia Galli Kienle*

*Scientific Institute H. S. Raffaele, Via Olgettina 58, 20132 Milano, Italy; and *Department of Medical Chemistry and Biochemistry, University of Milan, Via Saldini 50, 20133 Milano, Italy*

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Isocyclosporins are isomers of cyclosporins deriving from an N,O-acyl transfer occurring under acidic conditions. For the identification by ^{1}H NMR of isocyclosporins prepared in organic solvents the signal at 2.3 ppm assigned to three hydrogens of the $NHCH_3$ group is considered. Results reported here show that the ^{1}H NMR spectrum of isocyclosporin A prepared in aqueous acidic conditions lacks of the above signal and profoundly differs from that of isocyclosporin A prepared in organic solvents, despite the identity of the primary structures determined on the basis of mass spectrometric analysis.

INTRODUCTION

Cyclosporin A (CsA) is a neutral cyclic undecapeptide [cyclo(-MeBmt^1-Abu^2-Sar^3-MeLeu^4-Val^5-MeLeu^6-Ala^7-D-Ala^8-MeLeu^9-MeLeu^{10}-MeVal^{11}-)] with seven N-methylated amino acids well known as immunosuppressant. Under acidic conditions in organic solvent CsA and other known cyclosporins undergo rearrangement by N,O-acyl migration (*1–5*), with formation of isomers named isocyclosporins (isoCs). A signal at 2.3 ppm attributable to three hydrogens of the $NHCH_3$ group is reported to characterize the ^{1}H NMR spectra of isoCs B, C, D, E, G, and H (*3–5*). IsoCsA was described to be formed also under aqueous acidic conditions (*6*, *7*). Its identification was based upon the TLC and HPLC behavior in comparison with that of authentic isoCsA prepared with methanesulphonic acid according to Rüegger *et al.* (*1*). Formation of isoCsA under aqueous acidic conditions was also reported by us with the identification of the compound by FAB-MS/MS mass spectrometry (*8*, *9*). Isomerization of CsA in aqueous media may be particularly relevant in view of the reported *in vivo* isomerization of isoCsA to CsA. The aim of this work was to compare the ^{1}H NMR spectra of isoCsA prepared under these conditions with that of isoCsA obtained in organic solvent according to Rüegger *et al.* (*1*).

RESULTS AND DISCUSSION

The NCH_3 region of proton NMR spectra in $CDCl_3$ of CsA and of isoCsAs (Fig. 1) prepared both in organic solvent and in water are reported in Fig. 2. As already described (*10–12*) CsA exhibited one major conformation (Fig. 2a) and the chemical

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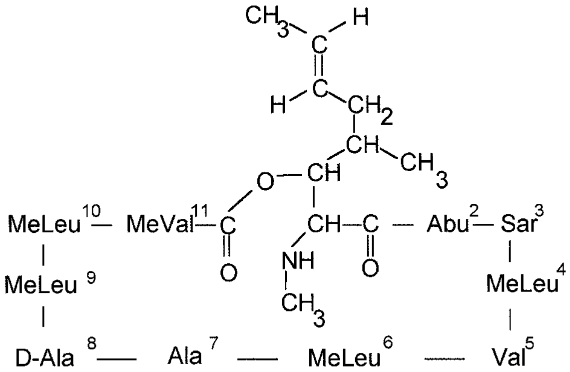

FIG. 1. IsoCsA.

shifts (Table 1) corresponded to those described previously (*11*). The spectrum of isoCsA prepared in organic solvent and purified on silica gel suggested the presence in $CDCl_3$ of few conformations (Fig. 2b). Nevertheless, one conformation appeared to prevail as suggested by the set of signals marked in Fig. 2b. Moreover the signal at 2.32 ppm (*s*, NCH_3) described to be characteristic of isoCs (*3–5*) was apparent. Fig. 2c shows the NCH_3 region of the spectrum of isoCsA prepared in aqueous HCl. Also for this compound one conformation appeared to be predominant but chemical shifts of the main signals did not correspond to those of the standard compound. In particular no signal at 2.3 ppm was observed. On the other hand FAB/MS-MS spectrum of isoCsA prepared under aqueous conditions was identical to that of standard isoCsA. Moreover, mass spectrum of isoCsA acetate prepared as described below was also indistinguishable from that of standard isoCsA acetate (Fig. 4). The downfield shift of the signal at 2.3 ppm in the ^{1}H NMR, as that reported for salts of amines (*14*) suggested protonation of the secondary amino group which instead appears as a free base in standard isoCsA. Protonation was accompanied by the shift of all other $CONCH_3$ signals, which suggested modification of the conformation. In order to confirm this hypothesis aliquots of the $CDCl_3$ solution of standard isoCsA were treated as described in Fig. 3 and chemical shifts of the NCH_3 hydrogens were considered. When standard isoCsA was either dissolved in an acidic medium (Fig. 3A) or eluted from an HPLC column (Fig. 3B) under conditions used for the purification of isoCsA prepared in aqueous medium, signals characteristic of standard isoCsA were absent in the ^{1}H NMR spectrum while main signals corresponded well to those observed for isoCsA prepared in aqueous medium as reported in Table 1, samples A and B. The same was shown to occur (Table 1, sample C) when a $CDCl_3$ solution of standard isoCsA was evaporated to dryness (Fig. 3C) followed by dissolution in $CDCl_3$ and registration of the NMR spectrum. The observed modifications were not attributable to low stability of isoCsA solutions in $CDCl_3$ because the NMR spectrum of standard isoCsA stored in $CDCl_3$ at 4°C for 7 days (Figs. 2d and 3D) suggested partial transformation of

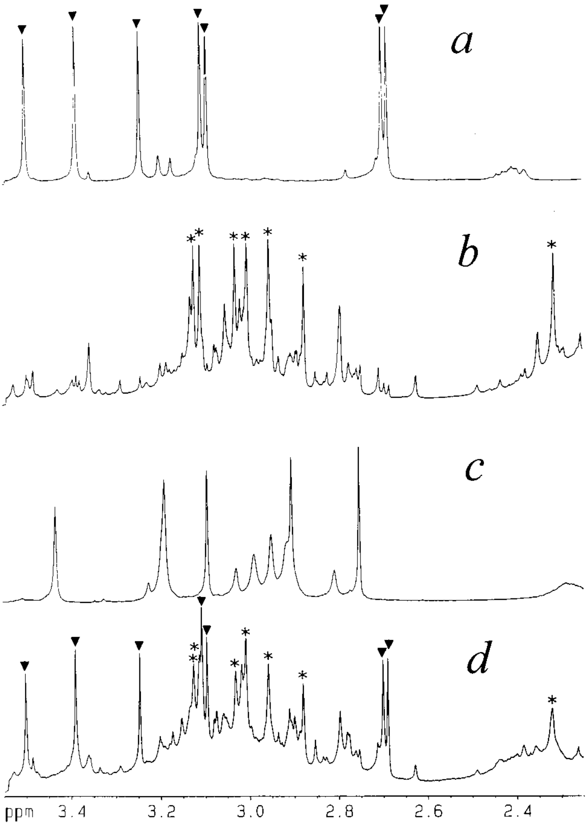

FIG. 2. NCH_3 region of proton NMR spectra of CsA and isoCsA. (a) Standard CsA; (b) standard isoCsA; (c) isoCsA prepared in aqueous medium; (d) standard isoCsA stored in $CDCl_3$ at 4°C for 7 days. ▼ and ∗ correspond to signals in (a) and (b), respectively.

isoCsA to CsA but showed no detectable signals at chemical shifts of isoCsA prepared in aqueous HCl (Table 1, sample D). Modifications may be rather justified by protonation of isoCsA. This may be true also for sample C in Table 1 due to the H^+ presence of $CDCl_3$ used as the solvent. Even if low this acidity may be sufficient for protonation of the secondary amino group of the analyzed isoCsA. Results obtained here may help to interpret previous reported ^1H NMR data, indicating multiplets instead of singlets in the 2.7–3.4 ppm region for the $CONCH_3$ signals of isoCs B, C, D, G, and H (3–5). Singlets, as expected, were instead reported for isoCsE (5). NHCO protons in the ^1H NMR spectrum of standard isoCsA (doublets in the 7.2–8 ppm region) confirmed the presence of a main conformation while signals in the spectrum of isoCsA prepared in aqueous medium were complex.

In conclusion the reported results demonstrate that isoCsA obtained by acidic