Synthesis of the Main Metabolite (OL-17) of Cyclosporin A

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Acetyl-cyclosporin A (2) was subjected to the conditions of allylic radical bromination to give 3. Exchange of the bromide by acetoxy led to 4 which was hydrolyzed to the known metabolite OL-17 (5) of cyclosporin A (1).

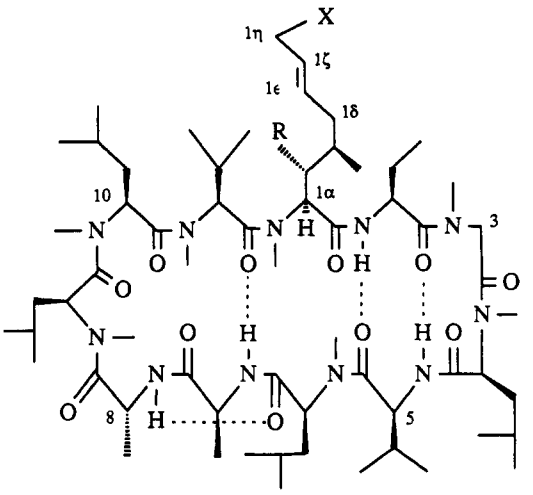
Cyclosporin A[1] (1), the active ingredient of Sandimmune, is a powerful immunosuppressant[2] preventing organ allograft rejections in animals[3] and humans.[4] It is available from natural sources[5] or through total synthesis.[6] The mechanism of action[7] is based on the inhibition of the production of lymphokines such as interleukine-2 (IL-2). Mainly, these lymphokines are secreted by the activated T helper cells thus stimulating the clonal expansion of activated T cells. These in turn are capable of distinguishing self from nonself in their response against antigens presented to the immune system in association with major histocompatibility complex (MHC) class I or class II gene products. Although it is recognized that cyclosporin A inhibits the transcription of lymphokines, the exact mechanism[8] is not clear. Cyclosporin A binds tightly to cyclophilin,[9] the postulated receptor, which in all likelihood is identical with the enzyme peptidyl–prolyl cis–trans isomerase.[10] The cyclophilin–cyclosporin A complex in turn binds to and inhibits the Ca^{2+} and calmodulin-dependent phosphatase calcineurin.[11]

Cyclosporin A is metabolized both in animals and humans mainly to a cyclosporin in which the original allylic methyl group is oxidized to the corresponding allyl alcohol 5[12] (see Chart I).

We have attempted to mimic this metabolic pathway in vitro. It was envisaged that a radical-initiated allylic bromination[13] on a properly protected cyclosporin A derivative should lead to a new cyclosporin with the original allylic methyl group having been oxidized to a bromomethylene group. Subsequently, the allylic bromide would then be replaced by an oxygen-bearing, e.g., acetoxy, group. Finally, removal, if necessary, of the protecting groups would lead to the main metabolite of cyclosporin A.

The hydroxy group of cyclosporin A (1) was protected with acetic anhydride in pyridine in the presence of a catalytic amount of (dimethylamino)pyridine to form acetylcyclosporin A (2). This known compound[14] was treated with a slight excess of *N*-bromosuccinimide (NBS) in the presence of a catalytic amount of azobisisobutyronitrile in a carbon tetrachloride solution.[13] Unfortunately, it turned out to be impossible to follow the reaction with the aid of chromatographic tools (HPLC, TLC). We were not able to find ways and means readily allowing the distinction and separation of the newly formed allylic bromide 3 from acetylcyclosporin A (2). On the other hand, mass spectroscopy of the crude reaction mixture clearly indicated the presence of a bromination product along with starting material and product of bisbromination. Fortunately, NMR spectroscopy allowed the monitoring of the progress of the bromination reaction. The disappearance of the signal due to the allylic methyl group of cyclosporin A[15] near δ 1.6 ppm could be followed. In its place, the newly formed allylic methylene group bearing one bromide gave rise to a new signal in the vicinity of δ 3.9 ppm. From these observations it was concluded that the allylic bromination had taken a regioselective course

Chart I



1: R = OH; X = H

2: R = OAc; X = H

3: R = OAc; X = Br

4: R = X = OAc

5: R = X = OH

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as anticipated above. On a preparative scale, separation of the bromide 3 from acetylcyclosporin A (2) turned out to be very tedious indeed. In practice, the allylic bromination was carried out in the presence of 1.2 equiv of *N*-bromosuccinimide and the crude bromide 3 was, following workup and isolation, heated in the presence of tetrabutylammonium acetate and a catalytic amount of sodium iodide in ethyl methyl ketone at 60 °C, effectuating the exchange of the bromide by the acetate. The bisacetate 4 thus generated was readily purified and characterized. Separation from unreacted, less polar acetylcyclosporin A (2) was accomplished through chromatography on a silica gel column or preferably on a preparative RP-18 reversed-phase medium- to low-pressure column. The actual preservation of the trans configuration of the double bond of the bisacetate 4 was demonstrated by NMR double resonance experiments. Irradiation of a solution of 4 in deuterated benzene at δ 4.75 ppm had two effects: (1) the multiplet near δ 5.7 ppm assigned to, among others, one of the two vinyl protons collapsed to a doublet with a coupling constant J = 16 Hz in agreement with the presence of a trans double bond in 4. This observation allowed the differentiation of the signals due to the two vinyl protons and the assignment of the chemical shifts to their respective vinyl protons; and (2) the doublet at δ 1.03 ppm (J = 8 Hz) assigned to the methyl group of the alanine of amino acid 8 collapsed to a singlet. Although this experiment does not allow an unequivocal distinction between the two methyl signals of the alanine groups, additional irradiations established a correlation between all the signals due to the NH, α-CH, and the methyl groups of the two amino acids 7 and 8, respectively. The relative positions of the chemical shifts of the signals assigned to these two amino acids are in agreement with previous observations[15] made with cyclosporin A (1).

Transesterification in methanol in the presence of sodium methoxide completed the conversion of cyclosporin A (1) to OL-17 (5), the desired metabolite of cyclosporin A. This was obtained in 94% yield following column chromatography over a reversed-phase RP-18 column (see Experimental Section). The configuration of the double bond was determined for this product as well. With the aid of double-resonance NMR spectroscopy the double bond of 5 was found to be trans. Irradiation at 4.0 ppm caused the multiplet between 5.5 and 5.6 ppm to collapse to a doublet with a coupling constant of J = 16 Hz, compatible with the presence of a trans double bond.

The overall yield of 5 from 2, without purification of the bromide, was about 67%, not taking into account recovered starting material. With the larger amount of OL-17 (5) available via the sequence of reactions described above, we were able to obtain OL-17 in crystalline form with a melting point of 181–183 °C. The comparison of ^{1}H and ^{13}C NMR spectra of the synthetic product with the spectra[16] of a sample of human metabolite OL-17 revealed the identity of both compounds.

Experimental Section

General. Thin-layer chromatography (TLC) plates were developed in ethyl acetate saturated with water. High-pressure liquid chromatography (HPLC) analyses were carried out using a RP-18 reversed-phase column at 75 °C. The spectra were monitored at 204 nm. The mobile phase consisted of aqueous acetonitrile with the amount of water varying between 15 and 40%. In addition, the aqueous phase contained 1 mL of 85% phosphoric acid per 3.7 L. Unless listed otherwise nuclear magnetic resonance (NMR) spectra were measured in deuterated chloroform solution on a 360-MHz spectrometer with TMS as reference. The assignment of the chemical shift, e.g., 2.67 (2.70) [s, 3 H, ^{11}NCH$_3$] refers to the observed signal for the methyl group attached to the nitrogen of amino acid 11. Except for *N*-methyl groups chemical shifts of proton NMR spectra are listed only if they differ significantly from those of the starting material. The assignments of the signals are tentative and based on the chemical shifts observed for the corresponding signals of cyclosporin A which are added for convenience (2.70). For the complete spectra of cyclosporin A (1) see ref 15. When ^{13}C and proton NMR spectra were taken, correlations between proton and carbon signals were done.

Acetyl-η-bromocyclosporin A (3). A mixture of 25.0 g (20 mmol) of cyclosporin A acetate (2),[14] and 4.4 g (25 mmol) of *N*-bromosuccinimide, and 400 mg of commercial azobisisobutyronitrile in 250 mL of CCl_4 was heated to reflux for 2.5 h. The solvent was evaporated and replaced by ether and filtered. The filtrate was washed with water, dried over $MgSO_4$, and evaporated to dryness. The residue was chromatographed on silica gel with ether/ethyl acetate (4:1) to give 10.7 g (40%) of amorphous product which was crystallized from ether/hexane to yield 8.4 g (32%) of pure substance: mp 207–209 °C; m/z calcd for $C_{64}H_{112}BrN_{11}O_{13}$ 1323.8, found 1324.6 (M + 1); $[\alpha_D]$ = −237.0° (c = 0.271 in MeOH); NMR δ 2.02 [s, 3 H, CH_3COO], 2.65 (2.70) [s, 3 H, ^{10}NCH$_3$], 2.67 (2.70) [s, 3 H, ^{11}NCH$_3$], 3.10 (3.11) [s, 3 H, ^{4}NCH$_3$], 3.20 (3.11) [s, 3 H, ^{9}NCH$_3$], 3.25 (3.27) [s, 3 H, ^{6}NCH$_3$], 3.26 (3.39) [s, 3 H, ^{3}NCH$_3$], 3.45 (3.51) [s, 3 H, ^{1}NCH$_3$], 3.91 [m, 2 H, CH_2Br]. Later fractions of the chromatogram contained an additional 11.2 g (42%) of product bringing the total yield to about 82%.

η-Acetoxyacetylcyclosporin A (4). A mixture of 4.31 g (3.3 mmol) of the crude bromide 3, contaminated with an estimated (by NMR) 15–20% of acetylcyclosporin A (2) and of 2.1 g (8 mmol) of tetraethylammonium acetate tetrahydrate in 30 mL of methyl ethyl ketone containing a catalytic amount of NaI was heated in an oil bath at 105 °C for 3 h and then left at room temperature for 2 days. The solvent was diluted with methyl *tert*-butyl ether and washed with water and brine. The organic layer was dried over $MgSO_4$ and evaporated to leave 4.0 g of crude product which was purified on a RP-18 reversed-phase column (240 g) with methanol/water (85:15) to yield 3.07 g (72%) of the product. A sample of 2.6 g was crystallized from ether/hexane to give 2.1 g of crystalline product: mp 191–192 °C; m/z calcd for $C_{66}H_{115}N_{11}O_{15}$ 1301.9, found 1302.9 (M + 1); $[\alpha_D]$ = −238.5° (c = 0.432 in MeOH); NMR δ 2.01 [s, 3 H, CH_3COO], 2.03 [s, 3 H, CH_3COO], 2.65 (2.70) [s, 3 H, ^{10}NCH$_3$], 2.68 (2.70) [s, 3 H, ^{11}NCH$_3$], 3.09 (3.11) [s, 3 H, ^{4}NCH$_3$], 3.21 (3.11) [s, 3 H, ^{9}NCH$_3$], 3.24 (3.27) [s, 3 H, ^{6}NCH$_3$], 3.26 (3.39) [s, 3 H, ^{3}NCH$_3$], 3.45 (3.51) [s, 3 H, ^{1}NCH$_3$], 4.35–4.50 [m, 3 H, CH_2OAc + ^{7}CH], 5.50–5.60 [m, 2 H, CH=CH]; NMR (C_6D_6) δ 1.03 [d, J = 8 Hz, 3 H, ^{8}CH$_3$], 1.55 [d, J = 8 Hz, 3 H, ^{7}CH$_3$], 1.76 [s, 3 H, CH_3COO], 2.03 [s, 3 H, CH_3COO], 2.63 [s, 3 H, ^{10}NCH$_3$], 2.83 [s, 3 H, ^{11}NCH$_3$], 2.97 [s, 3 H, ^{4}NCH$_3$], 3.11 [s, 3 H, ^{9}NCH$_3$], 3.15 [s, 3 H, ^{6}NCH$_3$], 3.33 [s, 3 H, ^{3}NCH$_3$], 3.46 [s, 3 H, ^{1}NCH$_3$], 4.57 [m, 1 H, ^{7}CH], 4.70–4.80 [m, 3 H, CH_2OAc + ^{8}CH], 5.65–5.75 [m, 1 H, CH=C*H*CH$_2$OAc], 5.95–6.10 [m, 2 H, C*H*=CHCH$_2$OAc + CHOAc], 7.5 [d, J = 8 Hz, 1 H, ^{5}NH], 7.9 [d, J = 8 Hz, 1 H, ^{8}NH], 8.3 [d, J = 8 Hz, 1 H, ^{7}NH], 8.7 [d, J = 8 Hz, 1 H, ^{2}NH].

η-Hydroxycyclosporin A (5). A mixture of 1.72 g (1.3 mmol) of the bisacetate 4 in 75 mL of CH_3OH and a solution of 1.2 g

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of sodium in 50 mL of CH_3OH was kept at room temperature for 2.5 h. The solution was acidified with acetic acid. The solvent was evaporated under reduced pressure, and the residue was dissolved in methyl *tert*-butyl ether, washed sequentially with water, brine, and $NaHCO_3$ solution, dried over $MgSO_4$ and evaporated. The crude product (1.6 g) was eluted from a RP-18 column (240 g) with methanol/water (85:15) to give 1.5 g (94%) of pure product. A sample was crystallized from ether/hexane to give a crystalline product of mp 181–183 °C dec; *m/z* calcd. for $C_{62}H_{111}N_{11}O_{13}$ 1217.9, found 1218.9 (M + 1); $[\alpha_D] = -174.0°$ (*c* = 0.612 in MeOH); NMR δ 2.67 (2.70) [s, 3 H, $^{10}NCH_3$], 2.68 (2.70) [s, 3 H, $^{11}NCH_3$], 3.13 (3.11) [s, 3 H, $^{4}NCH_3$], 3.17 (3.11) [s, 3 H, $^{9}NCH_3$], 3.28 (3.27) [s, 3 H, $^{6}NCH_3$], 3.45 (3.39) [s, 3 H, $^{3}NCH_3$], 3.47 (3.51) [s, 3 H, $^{1}NCH_3$], 3.9–4.05 [m, 3 H, OCH + OCH_2], 5.50–5.70 [m, 3 H, CH=CH + ^{9}NCH]; ^{13}C NMR δ 9.90 (9.93) [2-γ], 15.49 (16.07) [7-β], **17.36 (16.76) [1-γ-CH_3]**, 17.84 (18.19) [8-β], 18.22 (18.48) [5-γ'], 18.70 (18.75) [11-γ'], 19.75 (19.81) [5-γ], 20.33 (20.26) [11-γ], 21.01 (21.18) [4-δ'], 21.35 (21.93) [6-δ'], 21.79 (21.86) [9-δ'], 23.46 (23.49) [4-δ], 23.56 (23.38) [10-δ'], 23.73 (23.85) [10-δ] and (23.74) [9-δ], 23.96 (23.87) [6-δ], 24.40 (24.55) [10-γ], 24.70 (24.70) [9-γ], 24.83 (24.90) [4-γ], 24.89 (25.40) [6-γ], 25.10 (25.06) [2-β], 29.62 (29.05) [11-β], 29.83 (29.65) [$^{9}NCH_3$], 30.02 (29.81) [$^{11}NCH_3$], 31.09 (29.83) [$^{10}NCH_3$], 31.30 (31.32) [$^{4}NCH_3$] and (31.17) [5-β], 31.47 (31.53) [$^{6}NCH_3$], **32.16 (35.63) [1-δ], 32.34 (33.97) [$^{1}NCH_3$], 33.58 (35.99) [1-γ]**, 36.06 (35.99) [4-β], 37.41 (37.41) [6-β], 39.11 (39.04) [9-β], 39.29 (39.40) [$^{3}NCH_3$], 40.53 (40.73) [10-β], 44.83 (45.20) [8-α], 48.03 (48.30) [9-α], 48.40 (48.69) [7-α], 48.75 (48.86) [2-α], 50.13 (50.37) [3-α], 54.68 (55.31) [6-α], 55.38 (55.39) [5-α], 55.55 (55.51) [4-α], 57.43 (57.54) [10-α], **58.24 (58.75) [1-α]**, 58.47 (57.93) [11-α], **63.41 (17.96) [1-η], 72.52 (74.74) [1-β], 130.75 (126.32) [1-ζ], 131.84 (129.68) [1-ε]**, 169.03, 170.63, 170.67, 170.98, 171.12, 171.22, 171.26, 172.93, 173.03, 173.33, 173.59 [11 C=O].

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Kinetics and Mechanism of the Pyridinolysis of 2,4,6-Trinitrophenyl Acetate and 2,4,6-Trinitrophenyl Methyl Carbonate

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The title reactions are subject to a kinetic study in aqueous solution at 25.0° C, ionic strength 0.2 M. The reactions are first order in both the substrate and the free base pyridine. The Brönsted-type plots obtained are nonlinear with slopes $\beta_1 = 0.2$ and $\beta_2 = 0.8$ at high and low basicities of the pyridines, respectively, for both substrates. The pK_a values at the Brönsted breaks ($pK_a°$) are 5.0 and 6.5 for the acetate (TNPA) and the carbonate (TNPMC), respectively. The Brönsted curves can be better described by a two-step mechanism, with a tetrahedral intermediate, $T^{\pm}$, rather than a concerted process, although rigorously the latter mechanism cannot be ruled out. The higher $pK_a°$ for the TNPMC reactions, relative to TNPA, is in agreement with the results found in the aminolysis of the dinitro derivatives and is explained by the increased amine nucleofugality from $T^{\pm}$ when Me is replaced by MeO in $T^{\pm}$. Little or no effect on $pK_a°$ is observed by substitution of the O-aryl O atom of TNPA by an S atom; this is attributed to the high instability of the intermediates $T^{\pm}$ involved. The larger rate constants obtained in the pyridinolysis of 2,4,6-trinitrophenyl thiolacetate compared to that of TNPA is explained by the softer character of the carbonyl center of the former substrate.

Introduction

The aminolysis of aryl acetates and carbonates has been the subject of several mechanistic studies.[1-3] In most of these works a zwitterionic tetrahedral intermediate ($T^{\pm}$) in the reaction path has been postulated through nonlinear structure–reactivity correlations. The stability of $T^{\pm}$ has been found to be dependent on the nature and basicity of the amine moiety, the basicity of the aryloxy group, and the nature of the "acyl" group in $T^{\pm}$.

In the aminolysis of 2,4-dinitrophenyl acetate (DNPA), it was found that secondary alicyclic amines are expelled from $T^{\pm}$ faster than isobasic pyridines, indicating that the $T^{\pm}$ formed in the latter reactions is more stable than that produced in the former aminolysis.[4]

In the aminolysis of aryl acetates and carbonates the sensitivity of the rate of expulsion of aryloxide ion from $T^{\pm}$ to its basicity has been assessed.[3,4] An equation derived for the reactions of aryl acetates predicts a rate of ca. 3×10^9 s^{-1} for 2,4-dinitrophenoxide ion ($DNPO^-$) expulsion from the corresponding $T^{\pm}$.[4] The value predicted for 2,4,6-trinitrophenoxide ion ($TNPO^-$) leaving is ca. 2×10^{11} s^{-1}, indicating a very unstable $T^{\pm}$ which should have a borderline existence.

It has been reported that in the aminolysis of diaryl carbonates electron-withdrawal from the "acyl" group in $T^{\pm}$ favors amine expulsion relative to the aryloxide ion leaving.[3] The same effect was found by comparison of the aminolyses of DNPA and 2,4-dinitrophenyl methyl carbonate (DNPMC): Replacement of the methyl group of $T^{\pm}$ by methoxy (of larger electron-withdrawing *inductive* effect) increases the nucleofugality of the amine from $T^{\pm}$ relative to $DNPO^-$, rendering the latter $T^{\pm}$ more unstable.[5] Similarly, the change of methyl to substituted aryl as the "acyl" group in the $T^{\pm}$ formed in the aminolysis of acyl

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