THE PREPARATION OF [2-DEUTERO-3-FLUORO-D-ALA^8]CYCLOSPORIN A BY DIRECTED BIOSYNTHESIS

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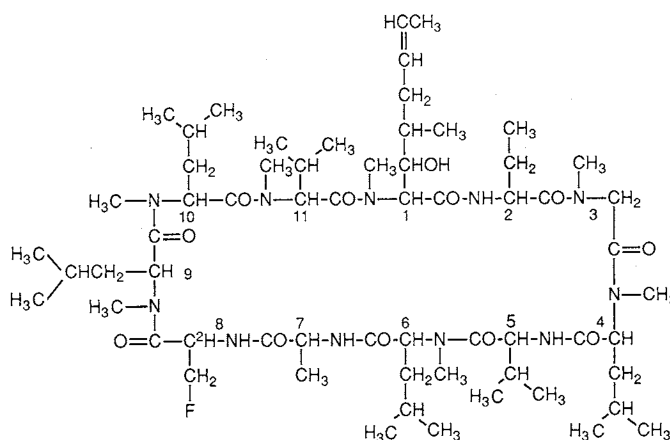
Several immunosuppressant cyclosporins modified at the D-Ala^8 position have been prepared by biosynthesis[1~3]. We have used this approach to produce functionality at the 8-position capable of synthetic derivatization. For this we required the biosynthesis of a 3-fluoro-D-Ala^8 (3-F-D-Ala^8) analog of cyclosporin A (CyA) which we were able to convert to derivatives of [D-Cys^8]CyA as described in the accompanying paper[4].

3-F-D-Ala was available to use as its 2-deutero analog (2-^{2}H-3-F-D-Ala) which several years ago was under study as a component of the antibacterial combination MK-641/MK-642[5,6]. 2-^{2}H-3-F-D-Ala (MK-641) is a potent irreversible inhibitor of alanine racemase[7,8]. We expected that it would inhibit the formation of D-Ala within the producing culture and be incorporated in its place to form a fluorinated cyclosporin analog given the close isosteric similarities of fluorine and hydrogen atoms. In fact, as an antibacterial agent 2-^{2}H-3-F-D-Ala was combined with the D-Ala-D-Ala ligase inhibitor pentizidone (MK-642) to prevent the incorporation of 2-^{2}H-3-F-D-Ala into the bacterial cell wall in place of D-Ala.

Tolypocladium inflatum MF-5080, NRRL-8040 was used to produce [2-^{2}H-3-F-D-Ala^8]CyA. A lyophile tube containing it was aseptically opened into 20 ml of seed medium containing glucose 50 g and malt extract 70 g per liter of distilled water in a 250-ml 3-baffle Erlenmeyer flask. The flask was incubated for 4 days on a rotary shaker (220 rpm) at 27°C. This seed was used to inoculate slant medium containing malt extract 20 g, yeast extract 4 g and agar 20 g. The slants were incubated at 27°C for 14 days after which time they were stored at 4°C until used. The entire contents of a slant were used to inoculate a 250-ml Erlenmeyer preculture flask of production medium (50 ml) containing glucose 40 g, casein peptone 10 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 2 g, $NaNO_3$ 3 g, KCl 0.5 g and $FeSO_4 \cdot 7H_2O$ 0.01 g. This preculture was incubated for 5 days at 27°C. Five ml of the preculture was used to inoculate 50 ml of production medium containing 5 mg/ml of 2-^{2}H-3-F-D-Ala in a 250-ml Erlenmeyer flask. The filter sterilized analog was added post-sterilization and prior to inoculation. The production flasks were incubated for 14 days with agitation (220 rpm) at 27°C. Following incubation, a combined 2.2 liters of fermentation broth was extracted with three 1.1 liters of methylene chloride. The cells were extracted with three 1.1 liters of acetone. The methylene chloride and acetone extracts were combined and taken to dryness under vacuum. A portion of the slightly oily residue was taken up in methanol and was analyzed by HPLC on a DuPont Zorbax ODS column (250 × 4.6 mm) at 60°C; mobile phase: acetonitrile - water (8 : 2); flow rate: 0.6 ml/minute; detection: UV-absorption at 210 nm. [2-^{2}H-3-F-D-



Structure of [2-^{2}H-3-fluoro-D-Ala^8]CyA.

Ala[8]]CyA exhibited a HPLC Rt of 0.94 relative to a CyA standard solution. No CyA was detected in the combined fermentation extract at a detection limit of 5% of the produced [2-^{2}H-3-F-D-Ala^8]CyA.

Initial purification of the fermentation extract residue was achieved by dissolving it in methanol - methylene chloride (1 : 1) and subjecting this solution to gel filtration chromatography on 200 ml of Sephadex LH-20. The chromatography was carried out in methanol at a flow rate of 5 ml/minute collecting one 8 ml fraction followed by forty 5 ml fractions. Fractions 22 through 26 were combined and concentrated to dryness. The residue was taken up in methanol and subjected to preparative HPLC chromatography on a DuPont Zorbax ODS column (2.1 × 25 cm) at 60°C using a mobile phase of acetonitrile - water (8 : 2) at a flow rate of 10 ml/minute monitoring the effluent stream by UV-absorption at 210 nm, collecting fractions based on the UV trace. Fractions 4 and 5 yielded pure [2-^{2}H-3-F-D-Ala^8]CyA (43 mg). FAB-MS *m/z* 1,200 (M^+); ^{1}H NMR ($CDCl_3$) δ 2.67 (s, CH_3N), 2.69 (s, CH_3N), 3.08 (s, CH_3N), 3.13 (s, CH_3N), 3.17 (d, J = 13.8 Hz, Sar^{3}αH′), 3.23 (s, CH_3N), 3.38 (s, CH_3N), 3.49 (s, CH_3N), 4.30 (dd, J = 8.7 and 46.2 Hz, Ala^{8}βH′), 4.46 (dd, J = 8.7 and 46.4 Hz, Ala^{8}βH″), 4.49 (dq, J = 7.3 Hz, Ala^{7}αH), 4.62 (dd, J = 8.4 and 9.9 Hz, Val^{5}αH), 4.70 (d, J = 13.7 Hz, Sar^{3}αH″), 4.94 (dd, J = 5.8 and 10.0 Hz, ML^{6}αH), 5.09 (d, J = 10.9 Hz, MV^{11}αH), 5.48 (d, J = 6.0 Hz, MB^{1}αH), 5.70 (dd, J = 4.4 and 10.9 Hz, ML^{9}αH), 7.06 (s, Ala^{8}NH), 7.46 (d, J = 8.3 Hz, Val^{5}NH), 7.70 (d, J = 7.3 Hz, Ala^{7}NH), 8.02 (d, J = 9.8 Hz, Abu^{2}NH). ^{13}C NMR ($CDCl_3$) δ 10.0 q (Abu^{2}γC), 16.1 q (Ala^{7}βC), 17.0 q (MB^{1}δC), 18.1 q (MB^{1}ηC), 18.5 q (Val^{5}γC), 18.8 q (MV^{11}γC), 20.0 q (Val^{5}γC), 20.5 q (MV^{11}γC), 21.2 q (ML^{4}δC), 21.8 q (ML^{6}δC), 22.0 q (ML^{9}δC), 23.6 q (ML^{10}δC), 23.6 q (ML^{4}δC), 23.9 q (ML^{6}δC), 23.9 q (ML^{10}δC), 24.0 q (ML^{9}δC), 24.2 d (ML^{10}γC), 24.5 d (ML^{9}γC), 24.9 d (ML^{4}γC), 25.0 t (Abu^{2}βC), 25.6 d (ML^{6}γC), 29.3 d (MV^{11}βC), 29.88 q (ML$^9NCH_3$), 29.94 q (ML$^{10}NCH_3$), 29.96 q (MV$^{11}NCH_3$), 31.2 d (Val^{5}βC), 31.4 q (ML$^4NCH_3$), 31.6 q (ML$^6NCH_3$), 34.1 q (MB$^1NCH_3$), 35.8 t (MB^{1}δC), 36.0 d (MB^{1}γC), 36.2 t (ML^{4}βC), 37.5 t (ML^{6}βC), 39.2 t (ML^{9}βC), 39.6 q (Sar^{3}NMe), 40.6 t (ML^{10}βC), 48.75 d (ML^{9}αC), 48.75 d (Ala^{7}αC), 48.83 d (Abu^{2}αC), 50.4 t (Sar^{3}αC), 55.3 d (ML^{6}αC), 55.45 d (Val^{5}αC), 55.49 d (ML^{4}αC), 57.6 d (ML^{10}αC), 57.9 d (MV^{11}αC), 59.0 d (MB^{1}αC), 74.8 d (MB^{1}βC), 81.9* d (Ala^{8}βC, $^1J_{C\text{-}F}$ = 177.2 Hz), 126.2 d (MB^{1}ζC), 129.5 d (MB^{1}εC), 169.8 s (CON), 169.9 s (2 × CON), 170.0* s (Ala^{8}CON, $^3J_{C\text{-}F}$ = 1.9 Hz), 170.1 s (CON), 171.1 s (CON), 171.53 s (CON), 171.56 s (CON), 173.4 s (CON), 173.59 s (CON), 173.61 s (CON). Resonances marked with an asterisk (*) were observed as doublets in the ^{1}H decoupled spectrum due to coupling with the ^{19}F nucleus. The multiplet t and s designations in these two cases denote methylene and quaternary carbons, respectively, the same as for other carbons. The abbreviations used are ML = *N*-methyl-Leu; MB = 4-(2-butenyl)-4,*N*-dimethyl-Thr; MV = *N*-methyl-Val and Abu = α-amino-butyric acid.

The structure of [2-^{2}H-3-F-D-Ala^8]CyA followed readily from its NMR spectroscopic and mass spectrometric properties. The FAB-MS was 19 mass units higher than that of CyA. ^{1}H (COSY and decoupling)[9] and ^{13}C NMR (^{1}H decoupled and attached proton test data[10] spectra) in $CDCl_3$ indicated a total of 61 carbons comprising 16 × CH_3, 6 × CH_2, 7 × CH, 7 × CH_3N, 1 × CH_2N, 9 × CHN, 1 × CH, 1 × CH_2F (d, J = 177.2 Hz), 2 × CH=, 11 × CON and 109 protons, 104 of which are bound to carbon and 5 active protons (4 × NH, 1 × OH). The data are consistent with the molecular formula $C_{62}H_{109}DN_{11}O_{12}F$ with the understanding that under the conditions of the experiment, the α-carbon of the 2-^{2}H-3-F-Ala residue was apparently not observable due to reduced intensity as a result of the absence of NOE and coupling to both D and ^{19}F.

Comparison of the ^{1}H and ^{13}C NMR spectra with those of CyA, relying on the definitive assignments of KESSLER *et al.*[11], readily established the position of incorporation. The methyl doublet, α-C*H* doublet of quartets and N*H* doublet resonances of the D-Ala^8 residue were replaced by an N*H* singlet at δ 7.06 and two doublet of doublets centered at δ 4.46 (J = 8.7 and 46.4 Hz) and δ 4.30 (J = 8.7 and 46.2 Hz), assigned to the nonequivalent CH_2F protons. Similarly in the ^{13}C NMR spectrum, the three D-Ala^8 carbon resonances of CyA were replaced by two doublets at 81.9 ($^1J_{C,F}$ = 177.1 Hz) and 170.0 ppm ($^3J_{C,F}$ = 1.9 Hz) and assigned to the *C*H_2F and *C*ON carbons, respectively, showing the expected coupling to fluorine. As noted above, the α-carbon carrying deuterium was not observed. The evidence is therefore consistent with substitution of the D-Ala^8 residue in CyA by 2-^{2}H-3-F-D-Ala.

The fact that [2-^{2}H-3-F-D-Ala^8]CyA was produced in the present study in the absence of detectable CyA is consistent with the hypothesis that D-Ala is formed in the producing organism and directly introduced into the biosynthetic pathway leading to CyA. Presumably the synthesis of D-Ala is blocked in *Tolypocladium inflatum* by 3-F-D-Ala's

inhibition of a pyridoxal-dependent alanine racemase in analogy with its action in bacteria.

[2-^{2}H-3-F-D-Ala^{8}]CyA is slightly more polar than CyA. Nonetheless its *in vitro* immunosuppressant activity closely approximates the nonfluorinated parent[4].

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