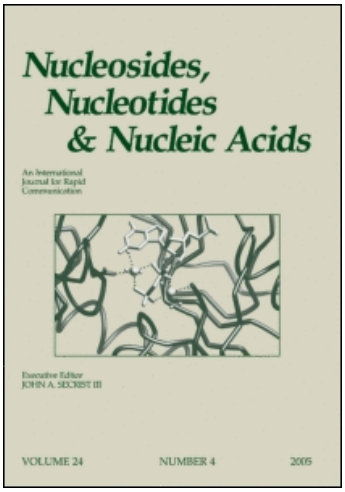

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RNA MODIFIED URIDINES.V.[1] AN IMPROVED SYNTHESIS OF 3-[3-(S)-AMINO-3-CARBOXYPROPYL]URIDINE (acp^3U) AND ITS 5'-PHOSPHATE.

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Abstract: The condensation of 2',3'-O-isopropylideneuridine sodium salt **3** with benzyl 2-(S)-benzyloxycarbonylamino- 4-iodobutyrate **2a** derived from L-homoserine, followed by the deprotection of **4a** in mild conditions gives the title nucleoside **1a** in 68% yield. The syntheses of acp^3U 3R-epimer **1b** and of acp^3U 5'-phosphate **6** are also described.

Introduction: 3-[3-(S)-amino-3-carboxypropyl]uridine **1a** (acp^3U or X) is present in the dihydrouridine loop (20, 20a or 20b position) and extra loop (47 position) of tRNAs from eukaryotes and prokaryotes, respectively.[2,3] The presence of acp^3U in the tRNAs sequence allows chemical modification of the biopolymer with photoreactive, fluorescent and paramagnetic labels[4] and use of such functionalized tRNAs for studies on the relation structure - function of tRNAs or ribosome topography.[4] The pathway of acp^3U biosynthesis is well known,[5] but its biological function as yet remains unclear.[4]

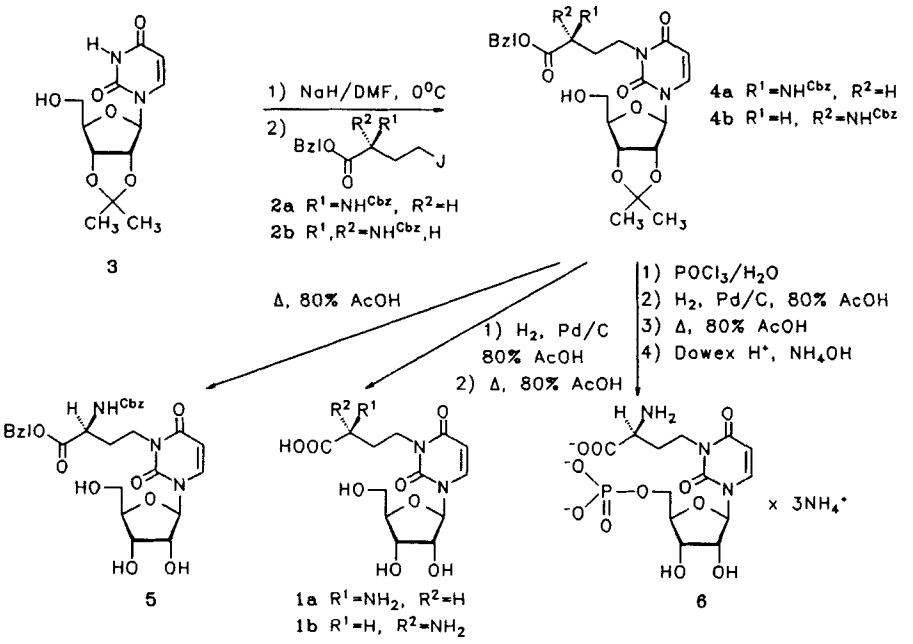
Results: The synthesis of acp^3U presented by Ohashi et al.,[6] Nishimura et al.,[5] and Seela et al.[7] was based on the alkylation of 2',3'-O-isopropylideneuridine sodium or potassium salt with ethyl 2-benzamido-4-bromobutyrate, followed by deprotection of the condensation product with concentrated hydrochloric acid. Drastic deprotection conditions caused, however, substantial degradation of the final product **1a**.

Tsuchida et al.[8] obtained **1a** in a moderate yield by condensation of 2',3'-O-isopropylidene-3-(2-formylethyl)-uridine, 2-picolinamide-1-oxide, cyclohexyl isocyanide and acetic acid.

In our approach to acp^3U synthesis we take advantage of the protection of the α-amino acid constituent with easily removable (by hydrogenolysis) blocking groups.

Thus, L-homoserine was transformed into benzyl 2-(S)-benzyloxycarbonylamino-4-O-(p-toluenesulfonyl) butyrate according to the described procedure[9] and the tosyl group was then exchanged into the iodo substituent to give **2a**.

The condensation of **2a** with 2',3'-O-isopropylideneuridine sodium salt in DMF gave the protected nucleoside **4a** in 84% yield (Scheme 1).



Scheme 1

The spectral data for **4a** fully confirm the N-3 substitution of uridine with the α-amino acid component. Hydrogenolysis of benzyl (Bzl) and N-benzyloxycarbonyl (Cbz) groups of **4a** proceeded in almost quantitative yield, but only in 80% acetic acid (10% Pd/C as catalyst, atmospheric pressure). Final deprotection of cis-diol function of ribose moiety produced nucleoside **1a**. All spectral data of **1a** are identical with those reported by other groups for synthetic acp^3U,[5,6,7] as well as for the natural product.[3,10]

To confirm an optical purity of **4a** compound **2b** was coupled with **3** in the previously described conditions, to give an equimolar mixture of diastereoisomers **4a** and **4b**, which were subsequently separated by HPLC. Only a minute difference of the chemical shifts can be noticed in the ^1H NMR spectrum of the equimolar mixture of **4a** and **4b**, for the H-5 and H-1' protons ($\Delta\delta_{H\text{-}5}$= 3.6 Hz, $\Delta\delta_{H\text{-}1}$= 9.1 Hz at 300 MHz).

The partially protected nucleosides **4a** and **4b** were deblocked following the previously described procedure to give **1a** and **1b**, respectively. Neither ^1H NMR nor CD and ORD spectra of **1a** and **1b** show any significant differences, to be used for an analytical purpose. On the other hand diastereoisomers **1a** and **1b** can be distinguished by HPLC technique. Under the conditions we have found as optimal (see Experimental), ΔR_T of **1a** and **1b** falls, however, in the range of half a minute only.[11]

According to the spectral and HPLC data neither condensation nor deprotection conditions cause any epimerization of the α-amino acid side chain.

The selective deprotection of the cis-diol function of **4a** with boiling 80% acetic acid affords nucleoside **5**, which was subsequently used for the synthesis of oligonucleotides by the phosphotriester method.[13]

The reaction of partially protected nucleoside **4a** with phosphorus oxychloride in the presence of a small amount of water[14] (Scheme 1), followed by the previously described deprotection and purification of the crude product by paper chromatography gave 5'-phosphate **6** in 40% yield (^{31}P NMR δ = 4.04 ppm). UV, CD, ORD and electrophoresis data for **6** are identical with those reported previously.[7]

This improved procedure was also applied for the synthesis of 1-methyl-3-[3-(S)-amino-3-carboxypropyl]pseudouridine ($m^1acp^3\psi$) which was identified in 17S and 18S rRNA hydrolyzates.[15,16]

Experimental:

^{1}H NMR spectra were determined on TESLA BS 467 (60 MHz) and TESLA BS 587A FT (80 MHz) spectrometers with TMS as the internal standard and on BRUKER MSL (300 MHz) with TMS or DSS as the external standards. Abbreviations: s-singlet, d-dublet, t-triplet, m-multiplet, br-broad.

Electron impact mass spectra (MS) were measured on a GS MS LKB 2091 instrument at 70eV and 15eV and FAB mass spectra were measured on an ADM 604 instrument.

CD and ORD measurements were made using JASCO J-20 Automatic Recording Spectropolarimeter at room temperature with 10 mm cell.

High-performance liquid chromatography (HPLC) was carried out on LDC/Milton Roy equipped with a UV spectroMonitor 3100, λ_{max}=254nm. a) Ultrasphere SI (BECKMAN) 5 μm stainless steel column (250 x 10 mm), hexane/ethyl acetate 55/45 v/v, flow rate 3.5 mL/min, b) ODS C-18 (VYDAC) stainless steel column (250 x 4.6 mm), 0.1M aqueous triethylammonium bicarbonate (pH 7.5), flow rate 1mL /min, c) ODS C-18 (Spherisorb) stainless steel column (250 x 4.6 mm), 0.02M $NH_4H_2PO_4$ /MeOH 95/5 v/v, flow rate 1 mL/min.

Thin layer chromatography (TLC) was performed on silica gel 60 F (Merck) plates in the solvent systems (v/v): A: hexane/ethyl acetate 2/1; B: chloroform/methanol 95/5; C: n-butanol/acetic acid/water 4/1/2; D: isopropanol/water/ ammonium hydroxide 7/2/1; E: chloroform/methanol 90/10; G: n-propanol/water/ammonium hydroxide 11/7/2; or on cellulose F (Merck) plates: F: isopropanol/water/ ammonium hydroxide 7/2/1.

Silica gel (70-230 mesh) was used for flash column chromatography. 3MM Whatman paper was applied for paper chromatography. Evaporations were carried out under reduced pressure and the bath temperature was kept below 40°C.

1.Benzyl 2-(S)-benzyloxycarbonylamino-4-iodobutyrate **2a**:

Benzyl 2-(S)-benzyloxycarbonylamino-4-O-(p-toluenesulfo-nyl) butyrate[9] (0.82g, 1.65mmol) [further purified by silica gel column chromatography, hexane/ethyl acetate 5/1, R_f=0.29 (A)] was refluxed in anhydrous acetone

(20mL), with potassium iodide (0.5g, 3mmol) for 4 hr. Inorganic salts were filtered off, the filtrate was concentrated to an oil and dissolved in chloroform (100mL). The organic solution was washed with water (20mL), dried over anhydrous $MgSO_4$ and concentrated to dryness. The crude product **2a**, (0.67g, 90% yield) was used directly in the next reaction. R_f=0.5 (A); ^{1}H NMR (60MHz, $CDCl_3$/TMS) δ ppm : 7.26 (br s, 10H, aromatic), 5.63 (br s, 1H, NH), 5.10 (s, 2H, CH_2 of benzyl ester), 5.03 (s, 2H, CH_2 of N-Cbz), 4.60-4.26 (m, 1H, N-CH), 3.06 (t, J=7Hz, 2H, N-$\underline{CH_2}$ -CH_2), 2.46-2.18 (m, 2H, N-CH_2-$\underline{CH_2}$).

2.Benzyl 2-(R,S)-benzyloxycarbonylamino-4-iodobutyrate **2b**:
Compound **2b** (1.34g) was obtained from D,L-homoserine (1.2g) according to the procedure described under heading 1.

3. 2',3'-O-isopropylidene-3-[3-(S)-benzyloxycarbonylamino-3-benzyloxy carbonylpropyl]uridine **4a**:
To a cooled (0°C) solution of 2',3'-O-isopropylideneuridine (382mg, 1.35mmol) in anhydrous N,N-dimethylformamide (15mL), NaH (33mg, 1.35mmol) was added and the reaction mixture was stirred for 30min. It was then allowed to warm up to room temperature and a solution of **2a** (0.67g, 1.5mmol) in N,N-dimethylformamide (5mL) was added dropwise into the reaction mixture. The reaction mixture was heated for 3 hr at 60-80°C and kept for 20 hr at room temperature. After evaporation of the solvent, the residue was coevaporated twice with toluene and dissolved in chloroform (100mL). The organic layer was washed with water (20mL), dried over $MgSO_4$ and evaporated. The residue was chromatographed on silica gel column with chloroform /methanol (99/1) to give 0.63g of **4a** (84% yield). R_f=0.33 (B); HPLC (a) R_T=34.47min; ^{1}H NMR (300 MHz, $CDCl_3$/TMS) δ ppm (after exchange with D_2O): 7.47-7.43 (m, 11H, aromatic,H-6), 5.79 (d, J=8.0 Hz, 1H, H-5), 5.61 (d, J=2.48 Hz, 1H, H-1'), 5.24 (br s, 4H, CH_2 of benzyl and N-Cbz groups), 5.18 (br s, 1H, H-2'), 5.04 (dd, $J_{2',3'}$=3.5Hz, $J_{3',4'}$=6.4 Hz, 1H, H-3'), 4.64 (t, J=5.7 Hz, 1H, N-CH), 4.40 (m, 1H, H-4'), 4.22-4.11 (m, 2H, N-$\underline{CH_2}$-CH_2), 3.99 (dd, $J_{4',5'}$=2.5 Hz, $J_{5',5''}$=12.1 Hz, 1H, H-5'), 3.92 (dd, $J_{4',5''}$=4.1Hz, $J_{5',5''}$=12.1 Hz, 1H, H-5''), 2.40-2.27 (m, 2H, N-CH_2-$\underline{CH_2}$), 1.69 (s, 3H, CH_3), 1.47 (s, 3H, CH_3); MS (70eV) m/z: 609(M, 0.43%), 594 (M-15, 0.76%), 518 (M-91, 0.76%), 474 (M-135, 2.19%), 366 (26.42%), 302 (12%), 267 (17.10%), 254 (14.03%); UV (MeOH); λ_{max}=208nm (ϵ=19x 10^3) and 265nm (ϵ=6.7x10^3).

4. 2',3'-O-isopropylidene-3-[3-(R,S)-benzyloxycarbonylamino-3-benzyloxy carbonylpropyl]uridine **4a and 4b**:
Following the procedure described under heading 3, 2',3'-O-isopropylidene uridine (284mg,1mmol) was condensed with **2b** (384mg,1.1mmol), to give a mixture of the diastereoisomers **4a** and **4b** (480mg, 79% yield). The sample of the mixture (80mg) was separated by HPLC (a) giving **4a**: R_T=34.47min (27mg,27% yield) and **4b**: R_T=36.63min (31mg,31% yield); Data for **4b**: R_f=0.33 (B); UV (MeOH) λ_{max}=208nm (ϵ=19x10^3) and 265nm (ϵ=6.7x10^3); MS (70eV) m/z: 609 (M, 0.50%), 594 (M -15, 0.75%), 518 (M-91, 0.95%), 474

(M -135, 3.0%), 366 (26.40%), 302 (10.08%), 267 (17.20%), 254 (15.05%); H^1NMR (300MHz, $CDCl_3$/TMS) δ ppm (after exchange with D_2O): 7.47-7.43 (m, 11H, aromatic, H-6), 5.80 (d, J=8.0 Hz, 1H, H-5, 5.64 (d, $J_{1',2'}$=2.75 Hz, 1H, H-1'), 5.24 (br s, 4H, CH_2 of benzyl and Cbz groups), 5.18 (br s, 1H, H-2'), 5.04 (dd, $J_{2',3'}$=3.6 Hz, $J_{2',3'}$=6.0 Hz, 1H, H-3'), 4.64 (t, J=5.7 Hz, 1H, N-CH), 4.40 (m, 1H, H-4'), 4.22-4.11 (m, 2H, N-$\underline{CH_2}$-CH_2), 3.98 (dd, $J_{4',5'}$=3.8 Hz, $J_{5',5''}$=12.1 Hz, 1H, H-5'), 3.92 (dd, $J_{4',5''}$=4.5 Hz, $J_{5',5''}$=12.1 Hz, 1H, H-5''), 2.40-2.26 (m, 2H, N-CH_2-$\underline{CH_2}$), 1.69 (s, 3H, CH_3), 1.47 (s, 3H, CH_3).

5.Deprotection of **4a** into **1a**. 3-[3-(S)-amino-3-carboxypropyl]uridine **1a**: To the stirred solution of **4a** (200mg, 0.33mmol) in 80% acetic acid (20mL), 10%Pd/C (50mg) was added. Hydrogen was passed through the reaction mixture for 1.5 hr at room temperature. The catalyst was filtered off and washed with 80% acetic acid. The reaction mixture was refluxed for 1 hr, the solvent was removed in vacuo and the residue was coevaporated several times with water. The water solution was poured on a Dowex H^+ (50X1 50-100 mesh, 3mL) column and it was washed with water to neutral pH of the eluate. The product was eluted with 10% NH_4OH (20mL) and finally purified by Whatman 3MM paper chromatography with isopropanol/water/ammonium hydroxide 7/2/1. **1a** (92mg, 81% yield) R_f=0.24(C), R_f=24(D), $R_f$cellulose=0.38(F) with positive ninhydrin test. HPLC(b) R_T=6.14min.; UV (H_2O) λ_{max}=265nm (ε=7.7x10^3), λ_{min}=233nm; MS of tetra TMS derivative, m/z 70eV: 634 (M+1, 5.06%), 618 (11.02%) 516 (55.76%), 461 (48.16%), 349 (15.46%), 259 (100%), 217 (86.31%); 15eV: 634 (M+1, 14.82%), 633 (M, 9.68%), 618 (19.66%), 516 (100%), 499 (23.75%), 498 (54.31%), 461 (85.62%), 349 (18.00%), 259 (96.67%); ^{1}H NMR (300MHz, D_2O/DSS) δ ppm: 7.90 (d, $J_{5,6}$=8.1 Hz, 1H, H-6), 5.99 (d, $J_{5,6}$=8.1 Hz, 1H, H-5), 5.95 (d, $J_{1',2'}$=4.0 Hz, 1H, H-1'), 4.39 (t, J=4.0 Hz, 1H, H-2'), 4.26 (t, J=5.4 Hz, 1H, H-3'), 4.17 (m, 1H, H-4'), 4.10 (t, J=6.7 Hz, 2H, N-$\underline{CH_2}$-CH_2), 3.96 (dd, $J_{4',5'}$=2.8 Hz, $J_{5',5''}$=12.8 Hz, 1H, H-5'), 3.84 (dd, $J_{4',5''}$=4.4 Hz, $J_{5',5''}$=12.8 Hz, 1H,H-5''), 3.70 (t, J=6.3 Hz, 1H, N-CH), 2.23 (m, 2H, N-CH_2-$\underline{CH_2}$); CD (H_2O): λ_{max}=220nm ([Θ]=-6.2x10^3) and 265nm ([Θ]=9.6x10^3), $\lambda_{crossover}$=249nm; ORD (H_2O): λ_{max}=248nm ([φ]=-7.0x10^3) and 276nm ([φ]=7.05x10^3), $\lambda_{crossover}$=262nm.

6. Deprotection of **4b** into **1b**. 3-[3-(R)-amino-3-carboxypropyl]uridine **1b**: According to the procedure described for the deprotection of **4a** (heading 5) nucleoside **4b** (30mg, 0.049mmol) was transformed into **1b** (12mg, 71%yield); R_f=0.24(C), R_f=0.24(D), $R_f$cellulose=0.38(F), positive ninhydrin test; HPLC(b) R_T=5.87min; UV(MeOH) λ_{max}=265nm (e=6.76x10^3); MS of tetra TMS derivative, m/z 70eV: 633(M, 3.55%), 618 (12.05%), 516 (48.03%), 499 (13.68%), 498 (27.37%), 461 (48.22%), 349 (20.80%), 259 (100%); m/z 15eV: 634 (M+1, 2.03%)/, 633 (9.50%), 618 (19.66), 516 (100%), 499 (23.75%), 498 (54.31%), 461 (88.02%), 349 (13.03%), 259 (82.03%); ^{1}H NMR (300MHz,D_2O/DSS) δ ppm: 7.90 (d, $J_{5,6}$=8.1 Hz, 1H, H-6), 5.99 (d, $J_{5,6}$=8.1 Hz, 1H, H-5), 5.95 (d, J1',2'=4.0 Hz, 1H, H-1'), 4.37 (t, J=4.1Hz, 1H, H-2'), 4.26 (t, J=5Hz,1H,H-3'), 4.17 (m, 1H, H-4'), 4.10 (t, J=6.7Hz, 2H,

N-$\underline{CH_2}$-CH_2), 3.96 (dd, $J_{4',5'}$=2.8Hz, $J_{5',5''}$= 12.8Hz, 1H, H-5'), 3.84 (dd, $J_{4',5''}$=4.4Hz, $J_{5',5''}$=12.8Hz, 1H, H-5''), 3.70 (t,J=6.3Hz, 1H,N-CH), 2.23 (m, 2H, N-CH_2-$\underline{CH_2}$); CD(H2O): λ_{max}=214nm ([Θ]=-6.6x10^3) and 265nm ([Θ]=7.7x10^3), $\lambda_{crossover}$=248nm; ORD (H_2O): λ_{max}=250nm ([φ]=-6.9x10^3) and 280nm ([φ]=6.2x10^3), $\lambda_{crossover}$=263nm.

7.Deprotection of **4a** into **5**. 3-[3-(S)-benzyloxycarbonylamino-3-benzyloxycarbonylpropyl] uridine **5**:
The solution of **4a** (58mg, 0.13mmol) in 80% acetic acid (2mL) was refluxed for 1.5 hr. The reaction mixture was evaporated, coevaporated three times with water and dried by several coevaporations with toluene. The crude product was purified by silica gel column chromatography (chloroform/methanol 98/2 v/v) to give **5** as a white foam (58mg, 77.6% yield). R_f=0.23(E); ^{1}H NMR (300MHz, CD_3OD/TMS) δ ppm: 8.03 (d, $J_{5,6}$=8.1 Hz, 1H, H-6), 7.36-7.25 (m, 10H, aromatic), 5.89 (d, $J_{1',2'}$=3.7 Hz, 1H, H-1'), 5.71 (d, $J_{5,6}$=8.1 Hz, 1H, H-5), 5.12 (s. 2H, CH_2 of benzyl group), 5.08 (s, 2H, CH_2 of Cbz group), 4.34-4.29 (m, 1H, N-CH), 4.20-4.12 (m, 2H, H-2', H-3'), 4.02 (t, J=7.1 Hz, 2H, N-$\underline{CH_2}$-CH_2), 4.02-3.99 (m, 1H, H-4'), 3.86 (dd, $J_{4',5'}$=2.5 Hz, $J_{5',5''}$=12.3 Hz, 1H, H-5'), 3.74 (dd, $J_{4',5''}$=3.1 Hz, $J_{5',5''}$=12.3 Hz, 1H, H-5''), 2.21-2.00 (m, 2H, N-CH_2-$\underline{CH_2}$).

8. Phosphorylation of **4a** into 5'-phosphate of 3-[3-(S)-amino-3-carboxy propyl]uridine, **6**:
240mg (0.39mmol) of **4a** was added to a mixture of freshly distilled phosphorus oxychloride (2mL) and 7.2μL of water. The mixture was stirred at 5°C for 20 hr. Hexane (100mL) was added and the precipitated product of condensation was collected by centrifugation. It was dissolved in 80% acetic acid (10mL) and 10% Pd/C (20mg) was added. Hydrogen was passed through the reaction mixture for 1 hr at room temperature. The catalyst was filtered off and the filtrate was refluxed for 1 hr. The solvent was lyophilized and the residue dissolved in water, poured on a Dowex H^+(50W X 8) column and eluted with 10% aqueous ammonia. The crude product was purified by paper chromatography with n-propanol/ water /ammonium hydroxide 11/7/2 system to give **6** (73mg, 40% yield). R_f=0.36(G), [$R_f$acp^{3}U=0.5(G)]; HPLC(c) R_T=3.64 min, [R_T5'pU =3.71 min]; ^{31}P NMR δ=4.04ppm; LSIMS (thioglycerol) positive ions: 448 (M+Na), 470 (M+2Na-H), 492 (M+3Na-2H); negative ions: 424 (M-H), 446 (M+Na-2H), 468 (M+2Na-3H).

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