the trans isomer 2 and the weakest inhibitor is the cis isomer 3. The order of activity as inhibitors of HMT appears to correlate with the ability of these drugs to block the action of histamine on the histamine H_1 receptor.[5,6] In contrast, the ability to potentiate the effects of HMT by these drugs appears to be completely opposite to their inhibitory effects on HMT and their antagonistic effects on the histamine receptor.

Since the double-reciprocal plots described above showed linear competitive kinetics (no increase in V_{max}), it can be assumed that the "activation" of HMT by these drugs results only from diminishing the inhibition produced at higher concentrations of histamine. The potentiation of HMT by these compounds, therefore, is not a true "activation" of the enzyme, but rather reversal of substrate inhibition. This substrate inhibition may be the result of allosteric mechanisms; however, neither the present work nor that of Taylor and Snyder provides definitive evidence for allosteric mechanisms.[3]

In summary, it can be concluded that a relationship exists between the HMT inhibitory effects of compounds 2 and 3 and their effects as histamine antagonists on the H_1 receptor. The trans isomer 2 is a better HMT inhibitor and a better histamine antagonist than the cis isomer 3. However, the cis isomer 3 is more active in potentiating HMT activity, whereas the trans isomer 2 shows little potentiating ability. This may indicate a difference in the conformational requirements associated with the inhibition and potentiation of HMT by antihistamines.

Experimental Section

Materials. SAM-$^{14}CH_3$ (New England Nuclear, 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/ml and stored at −20°F. SAM iodide (Sigma) was stored as a 0.01 *M* stock solution.

Tripelennamine was a gift from Dr. A. J. Plummer and compounds 2 and 3 were prepared by previously described procedures.[6]

Histamine *N*-Methyltransferase (HMT) Purification. HMT was purified from guinea pig brain (Pel-Freez Biologicals) according to the methods previously described by Brown, *et al.*[2] The enzyme was purified through the dialysis step resulting in a preparation which contained 15.2 mg of protein per milliliter. This represented a ninefold purification from the crude supernatant.

Measurement of Enzyme Activity. Kinetic experiments were carried out in 15-ml screw cap culture tubes and the basic incubation mixture contained the following components (in micromoles) added in this sequence: water, so that final volume was 0.25 ml; histamine (variable); inhibitor (variable); *S*-adenosyl-L-methionine (variable); 0.05 μCi of *S*-adenosyl-L-methionine-^{14}C; phosphate buffer, pH 7.40 (10); and the enzyme preparation (0.05 ml). The reaction was started by addition of enzyme and incubated for 15 min at 37°. The reaction was stopped by addition of 0.25 ml of 0.5 *M* borate buffer, pH 10.0, and the aqueous mixture extracted with 10 ml of toluene–isoamyl alcohol (1:1). After centrifugation, an aliquot (5 ml) of the organic phase was transferred to a scintillation vial, a dioxane-based phosphor solution (10 ml) added, and the radioactivity measured in a Beckman LS-150 scintillation spectrophotometer.

Data Processing. Reciprocal velocities were plotted graphically against reciprocals of the substrate concentrations. In all cases, for the substrate concentration ranges used, reasonably linear relationships were obtained.

Data fitting linear competitive inhibition patterns were fitted to eq 1 using a Hewlett-Packard 2100A digital computer and a Fortran IV program.[7,8]

$$v = VA/(K[1 + I/K_{is}] + A) \quad (1)$$

Acknowledgment. R. T. B. gratefully acknowledges partial support by a Health Science Advancement Award (FR06147) provided to the University of Kansas by the National Institutes of Health and a grant from the Research Corporation. P. E. H. gratefully acknowledges support by Grant GM 15477 from the National Institutes of Health, U. S. Public Health Service.

References

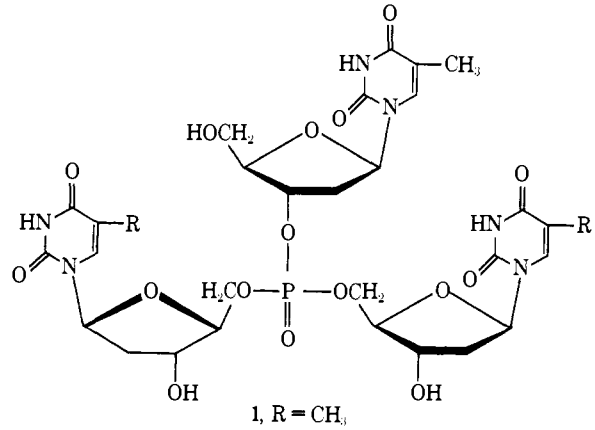
(1) R. W. Schayer, *Brit. J. Pharmacol. Chemother.*, 11, 472 (1956).
(2) D. D. Brown, R. Tomchick, and J. Axelrod, *J. Biol. Chem.*, 234, 2948 (1959).
(3) K. M. Taylor and S. H. Snyder, *Mol. Pharmacol.*, 8, 300 (1972).
(4) K. M. Taylor, *Biochem. Pharmacol.*, 22, 2775 (1973).
(5) P. E. Hanna, A. E. Ahmed, V. R. Grund, and R. L. Merriman, *J. Pharm. Sci.*, 62, 512 (1973).
(6) P. E. Hanna and A. E. Ahmed, *J. Med. Chem.*, 16, 963 (1973).
(7) W. W. Cleland, *Advan. Enzymol.*, 29, 1 (1967).
(8) W. W. Cleland, *Nature (London)*, 198, 463 (1963).

Synthesis of Some Trinucleoside Monophosphates of Biological Interest

Eric J. Norman and Joseph Nagyvary*

Texas A & M University, Department of Biochemistry and Biophysics, College Station, Texas 77843.
Received September 24, 1973

Synthesis and studies of some trinucleoside monophosphates were undertaken in the hope of finding a way of introducing nucleotide analogs into mammalian cells. Phosphotriesters of intermediary stability seemed suitable to achieve this goal, in spite of negative results reported for the diethyl FdUrd-5′-P.[1,†] Trinucleoside monophosphates resemble the nucleosides in that they are both neutral and possess free hydroxyl groups, but they also contain an internucleotide linkage. These compounds *per se* might exhibit interesting biological properties. So far, tri(uridine 5′-) phosphate and di(uridine 5′-) thymidine 3′-phosphate were the only compounds of this class obtained by a deliberate synthesis.[2] Here we describe the synthesis of di(thymidine 5′-) thymidine 3′-phosphate (1, $dThd_3$-P), bis(5-bromodeoxyuridine 5′-) thymidine 3′-phosphate [2, $(BrdUrd)_2dThd$-P], and bis(5-fluorodeoxyuridine 5′-) thymidine 3′-phosphate [3, $(FdUrd)_2dThd$-P].



1, R = CH_3
2, R = Br
3, R = F

Of the several synthetic approaches which we explored, the most successful one was the esterification of 5′-*O*-tritylthymidine 3′-phosphate (TrdThd-P) with an excess of unprotected nucleosides using triisopropylbenzenesulfonyl chloride as the condensing agent. After acidic removal of the trityl groups, the triesters 1, 2, and 3 were obtained by passing the mixtures through Dowex-1 resin, followed by silica gel chromatography. The generally poor

†Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; BrdUrd, 5-bromo-2-deoxyuridine; dThd, 2′-deoxythymidine; FdUrd-5-P, the corresponding nucleotide.

Table I. R_f Values of Synthesized Compounds and Standards

Compd	Paper chromatography		Electrophoresis[a]	
	Solvent A	Solvent B	pH 7.5	pH 4.0
dThd	0.585	0.521	0.0	n.t.
BrdUrd	0.58	0.55	n.t.	0.10
FdUrd	0.515	0.415	0.21	0.10
$dThd_3$-P	0.359	0.183	0.00	n.t.
$(BrdUrd)_2dThd$-P	0.358	0.155	0.12	0.08
$(FdUrd)_2dThd$-P	0.33	0.120	0.29	0.08
dThd-*p*dThd	0.375	0.14	0.38	0.72
BrdUrd-*p*dThd	0.339	0.02	0.45	0.80
FdUrd-*p*dThd	0.235	0.019	0.56	0.82

[a] Thymidine 3′-monophosphate pyridinium salt was used as a reference of 1.0. Electrophoresis was run for 1.5 hr at 2000 V. n.t. = no test.

yields are probably due to the steric barriers in introducing the third nucleoside moiety and, possibly, to the instability of the products under the conditions of the synthesis and purification. The formation of the 3′,3′,5′ isomer is even more difficult for steric reasons,[3] and only traces of this isomer would have been present in our products. The condensation of tritylthymidine with 3′-*O*-acetylthymidine 5′-phosphate did not produce any neutral phosphate ester. Elementary analyses and nmr spectra of **1**, **2**, and **3** are in accord with the assigned structures.

While triester **1** is quite stable at physiological pH, triester **3** is hydrolyzed to an extent of approximately 10% in 0.1 *M* $NaHCO_3$, pH 7.5, at 37° during 24 hr. This difference may be due to the low p*K* value of the FdUrd moiety resulting in the partial ionization of **3** at neutral pH which is apparent on electrophoresis. A certain degree of instability of dinucleoside monophosphates containing FdUrd was noted previously.[4] The most plausible mechanism underlying the hydrolysis of these compounds in neutral solution could involve the participation of the FUra anion. The analysis of the products following treatment of **2** and **3** with 1 *N* NaOH at 20° for 5 hr revealed the simultaneous appearance of thymidine and 5-halogenonucleoside (Table I).

As expected, triesters **1**, **2**, and **3** were resistant to the action of snake venom phosphodiesterase and spleen phosphodiesterase, in accordance with the behavior of other nucleotide triesters.[2,5] However, there is some evidence of possible enzymatic degradation of insecticide phosphotriesters *in vivo*,[6] which process might be applicable to other triesters.

Biological. The problem whether or not trinucleoside monophosphates can be similarly biodegradable was studied in the thymidine requiring strain of *Escherichia coli* 0191 Thd^-. It was found that triester **1** supported the growth of this mutant at high concentrations (100 μg/ml).

Triesters **2** and **3** exhibited moderate inhibition on mammalian cells in tissue culture, while **1** caused no inhibition.‡ On the basis of these findings, we assume that the triester is first hydrolyzed *via* a slow chemical process to diester which is then cleaved in a more rapid enzymatic step.[1] It seems that the first step of hydrolysis of the above triesters is too slow to serve our original purpose, *i.e.*, the direct introduction of a nucleoside 5′-phosphate into mammalian cells. Triester **3** was tested in a laboratory of Dr. Charles Heidelberger on Novikoff hepatoma (NS) cells and a FdUrd-resistant line (NSF) that lacks thymidine kinase. In the NS cells the triester was about 50–100 times less active than FdUrd; in the NSF cells the triester was inactive at 10^{-5} *M*. Even if the compound were taken up by the cells, the rate of its hydrolysis would not have yielded a critical concentration of the nucleotide analog. The slight inhibition produced by the triester may be due to a very slight cleavage to FUra or FdUrd. More labile phosphotriesters might still be useful in overcoming the resistance to FdUrd.

‡ Preliminary results of Dr. W. Kreis, Sloan-Kettering Institute, on P815 cells.

Experimental Section§

Di(thymidine 5′-) Thymidine 3′-Phosphate ($dThd_3$-P, 1). 5′-*O*-Tritylthymidine 3′-phosphate pyridinium salt (TrdThd-P, 375 mg, 0.52 mmol) and thymidine (363 mg, 1.5 mmol) were condensed in pyridine (5 ml) using triisopropylbenzenesulfonyl chloride (TPS, 735 mg, 2.42 mmol) at 20° for 12 hr. After evaporation, the mixture was distributed between $CHCl_3$ (75 ml) and water (25 ml), and the material present in the $CHCl_3$ phase was heated in 90% acetic acid at 100° for 30 min. The solution was through Dowex-1 (formate resin). The neutral fraction was chromatographed on a silica gel column (85 × 2.5 cm) in a *n*-BuOH–H_2O (85:15 v/v solvent B). Triester **1** was eluted at 600–800 ml (70 mg, 18%). Alternatively, pure **1** could also be obtained by passing the neutral fraction through a Sephadex G-10 column (55 × 3.4 cm) in 50% EtOH (V_e = 230–280 ml). After drying the freeze-dried powder [*Anal.* $C_{33}H_{39}N_6O_{16}P\cdot 1H_2O$ C, H, N, P] had mp 150° dec; uv and ORD (EtOH) identical with thymidine; nmr (DMSO-d_6) δ 2.28 (broad singlet, 9, CH_3), 6.5–6.7 (m, 3, H-1′), 7.87 (s, 2, H-6), and 8.08 (s, 1, H-6); uv λ_{max} (H_2O) 267 nm (ε 27,300).

Triester **1** was also prepared using 3′-*O*-acetylthymidine in place of thymidine. The removal of the acetyl groups with NH_3–MeOH was accompanied by hydrolysis of the triester, and the yield was small.

Bis(bromodeoxyuridine 5′-) Thymidine 3′-Phosphate [$(BrdUrd)_2dThd$-P, 2]. TrdThd-P (500 mg, 0.69 mmol) and 5-bromodeoxyuridine (638 mg, 2.08 mmol) were condensed using TPS (1.05 g, 3.5 mmol) and processed by the same procedure as described for **1**. Purification of the neutral fraction on silica gel column and paper chromatography in *n*-BuOH–H_2O (85:15) afforded 648 OD, 273 units (3.5%) [*Anal.* ($Br_2C_{28}H_{33}N_6O_{16}P$ + $1.5C_4H_9OH$) C, H, N]; nmr (DMSO-d_6) δ 2.23 (s, 3, CH_3), 6.45–6.75 (m, 3, H-1′), 7.79 (s, 2, H-6), and 8.11 (s, 1, H-6); λ_{max} (EtOH) 273 nm (ε 26,600, calcd).

Bis(5-fluorodeoxyuridine 5′-) Thymidine 3′-Phosphate [$(FdUrd)_2dThd$-P, 3]. TrdThd-P (375 mg, 0.52 mmol) and 5-fluorodeoxyuridine (368 mg, 1.5 mmol) were condensed using TPS (1.5 g, 5 mmol) according to the procedure described for **1**. After deblocking, triester **3** was isolated by chromatography on silica gel in solvent B (1600 OD, 266 units, 22%) [*Anal.* ($C_{28}F_2H_{33}N_6O_{16}P$ + $3H_2O$) C, H, N, P]; λ_{max} (EtOH) 266 nm (ε 30,000); nmr (DMSO-d_6) δ 2.25 (s, 3, CH_3), 6.5–6.8 (m, 3, H-1′), 8.13 (s, 1, H-6), and 8.20 (d, J = 6 Hz, 2, H-6).

A small aliquot of **3** (1 mg) was treated with 1 *N* NaOH at 20° for 5 hr and neutralized, and the products were analyzed by paper chromatography. In addition to a diester, about equal amounts of dThd and FdUrd were found.

Acknowledgments. We are grateful to Professor Charles Heidelberger and Dr. J. Rodney Parkhurst, McArdle Laboratory, University of Wisconsin, for testing our compounds against cultures of Novikoff hepatoma cells. We also thank Dr. J. C. Williams and Dr. G. A. O'Donovan for the bacteriological tests. This work was supported by a grant from the National Cancer Institute of the National Institutes of Health (CA 11389).

References

(1) K. L. Mukherjee and Ch. Heidelberger, *Cancer Res.*, **22**, 815 (1962). For abbreviations see *Biochemistry*, **9**, 4025 (1970).
(2) J. Nagyvary, *Biochemistry*, **5**, 1316 (1966).
(3) R. L. Letsinger and K. K. Ogilvie, *J. Amer. Chem. Soc.*, **91**, 12, 3350 (1969).

§ All compounds were analyzed for C, H, and N and gave results within ±0.4% of the theoretical values. Paper chromatograms were run on Whatman No. 3 MM paper in solvent A—30 parts solvent B, 5 parts 95% ethanol, and 5 parts 1 *M* ammonium acetate; solvent B—1-butanol–H_2O (85:15, v/v). Electrophoresis was performed in a Savant apparatus using 0.05 *M* sodium phosphate, pH 7.5, and 0.1 *M* CH_3COONH_4, pH 4.0. Nmr spectra were recorded on a Varian HA-100 spectrometer with TMS as the external standard.

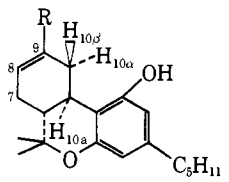
(4) A. Bloch, M. H. Fleysher, R. Thedford, R. J. Maue, and R. H. Hall, *J. Med. Chem.*, **9**, 886 (1966).
(5) P. S. Miller, K. N. Fang, N. S. Kondo, and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, **93**, 6657 (1971).
(6) N. P. B. Dudman and B. Zerner, *J. Amer. Chem. Soc.*, **95**, 3019 (1973).

Communications to the Editor

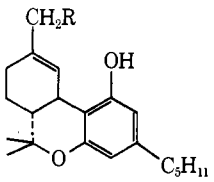
9-Nor-Δ^8-tetrahydrocannabinol, a Cannabinoid of Metabolic Interest

Sir:

Both of the psychotomimetically active components of marihuana, Δ^8-THC (**1a**) and Δ^9-THC (**2a**),[1] are metabolized to the biologically active 11-hydroxy compounds, **1b** and **2b**, respectively,[1,2] and a number of other less active or inactive oxygenated compounds.[2e,3] In both mice[3] and monkeys,[4] the pharmacological effects of **1b** are comparable to the parent compound **1a**. 11-Hydroxy-Δ^9-THC (**2b**) has been shown to parallel the activity of the parent compound **2a** in man[5] as well as in mice.[3] The greater potency of the 11-hydroxy metabolites over the parent compounds following intracerebral injection has led to speculation that these metabolites may be the active form of THC,[3] a postulate apparently supported by the observed delay in onset of certain behavioral effects of Δ^9-THC[6] in rats after giving the known metabolic inhibitor of Δ^9-THC,[7] SKF 525-A (2-dimethylaminoethyl-2,2-diphenylvalerate hydrochloride). An attempt to correlate behavioral effects in mice with brain concentration of Δ^9-THC or 11-hydroxy-Δ^9-THC was inconclusive when it was found that the levels of both substances paralleled the behavioral response.[8] The latter investigators also demonstrated that at 25 mg/kg of SKF-525a the brain levels of Δ^9-THC were only slightly increased whereas the levels of 11-hydroxy-Δ^9-THC were increased nearly threefold. This observation tends to cast doubt on the interpretations of some previous experiments employing SKF-525a and Δ^9-THC. However, it was found that infusion of 11-hydroxy-Δ^9-THC and Δ^9-THC showed no significant difference in potency and time of onset of cardiac effects and subjective "high" in human subjects.[2e] This suggests that the parent compound may be active itself. Also, a number of synthetic cannabinoid analogs which cannot be converted to 11-hydroxy metabolites have been found to have some pharmacologic properties in common with Δ^8- and Δ^9-THC.[9,†]



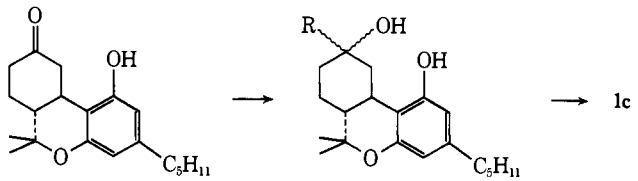
1a, R = CH_3
b, R = CH_2OH
c, R = H



2a, R = H
b, R = OH

The magnitude of the controversy over whether THC must first be metabolized at the 11 position to be active and the need for definitive evidence to resolve this controversy prompted us to synthesize 9-nor-Δ^8-THC (**1c**). It was reasoned that if the parent Δ^8-THC was active, then **1c** should be structurally similar enough to mimic its activity. However, if the bulk of the activity resided in **1b**, compound **1c** should be essentially inactive.

Thus, sodium borohydride reduction of 9-nor-9-oxohexahydrocannabinol (**3**)[10] gave a quantitative yield of 9-nor-9-hydroxyhexahydrocannabinol (**4a**) as a mixture of diastereomers.‡ Dehydration of **4a** (refluxing benzene, *p*-toluenesulfonic acid, 6 days) afforded an oily olefin§ (54% yield) purified by column chromatography on silica gel (5% ether in petroleum ether as eluent). By analogy to the dehydration of **4b** under similar conditions,[10] the thermodynamically more stable Δ^8 olefin[11] would be expected.



3 → **4a**, R = H; **b**, R = CH_3 → **1c**

Nmr spectra of this olefin confirm the structure as **1c**. The 60-MHz ($CDCl_3$) spectra of **1a** and **1c** are nearly identical except that **1c** lacks the vinyl methyl and there are two olefinic protons in **1c** of slightly different chemical shift from that of the single olefinic proton in **1a** (δ 5.78, multiplet for the two olefinic protons of **1c** *vs.* a broad absorption at δ 5.40 for **1a**).[10] This would appear to argue against the Δ^9 position for the olefinic bond, because in Δ^9-THC the olefinic proton lies in the deshielding region of the aromatic ring and absorbs at δ 6.32.[10] The close parallelism observed between the double-resonance studies of Δ^8-THC at 220 MHz[12] and those of **1c** (at 100 MHz) further rules out the Δ^9 (or Δ^7) position of the double bond. The double-resonance results are summarized in Table I for Δ^8-THC and **1c**. Two features of the 100-MHz nmr spectrum of **1c** are pertinent to the assignment of the position of the double bond.

First, the chemical shift of $H_{10\alpha}$ (δ 3.36) suggests that this proton is in an allylic position.[=] Secondly, $H_{10\alpha}$ is coupled to the H_9 olefinic proton,** thereby ruling out the Δ^7 position for the double bond.

Preliminary biological testing of **1c** was performed in dogs utilizing a previously reported procedure.[14] In unanesthetized dogs **1c** produced a prance-like placement of the feet, static ataxia, hyperreflexia, and a decrease in

†See compounds **16** and **32**. Also see compounds LII, LIII, LIV, LXI, and LI of ref 1b.

‡ Physical data for **4a**: ir ($CHCl_3$) 3590, 3420, 1622, and 1579 cm^{-1}; mass spectrum (70 eV) *m/e* 318 (parent), 300, 261, 257, 193; mp 178–179°. *Anal.* Calcd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50. Found: C, 75.37; H, 9.66.

§Physical data for **1c**: mass spectrum (70 eV) *m/e* 300 (parent), 257, 244, 231. *Anal.* Calcd for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39. Found: C, 79.65; H, 9.42.

= $H_{10\alpha}$ in hexahydrocannabinol is at δ 2.89[12] and in Δ^7-THC is at δ 2.95.[13]

**$J_{9,10\alpha}$ was not determined; however, irradiation of the olefinic absorption resulted in the absorption for $H_{10\alpha}$ collapsing from a doublet of multiplets into a resolved doublet of doublets. Similarly, irradiation of $H_{10\alpha}$ caused considerable sharpening of the olefinic absorption.