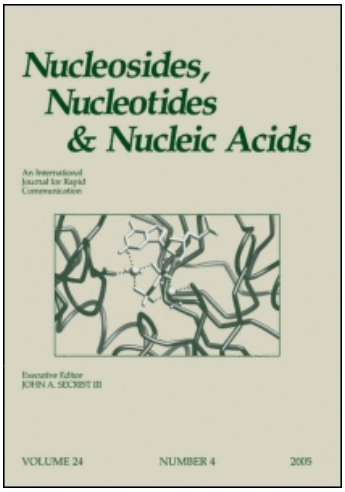

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A Simple Method for Synthesis of Spongosine, Azaspongosine, and Their Antiplatelet Effects

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A SIMPLE METHOD FOR SYNTHESIS OF SPONGOSINE, AZASPONGOSINE, AND THEIR ANTIPLATELET EFFECTS

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Abstract

Reaction of 2-ethylthioadenine (**1**) with protected ribose (**2**) in the presence of stannic chloride gave 2-ethylthioadenosine (**4**). Oxidation of **5** with potassium permanganate yielded the corresponding sulfone (**6**) which furnished spongosine (**7**) after treatment with sodium methoxide. Similarly, reactions of 7-amino-5-ethylthio-1,2,3-triazolo[4,5-d]pyrimidine (**8**) with the ribose (**2**) gave 8-azaspongosine (**13**). The compounds (**4**) and **7** demonstrated potent antiaggregatory effects both in human platelet-rich plasma and whole blood, whereas, the aza analog (**13**) showed no inhibitory activity on platelet aggregation. Both (**4**) and (**7**) inhibit platelet aggregation in the presence of adenosine deaminase, whereas, adenosine is non-inhibitory, suggesting that analogs (**4**) and (**7**) are poor substrates for adenosine deaminase.

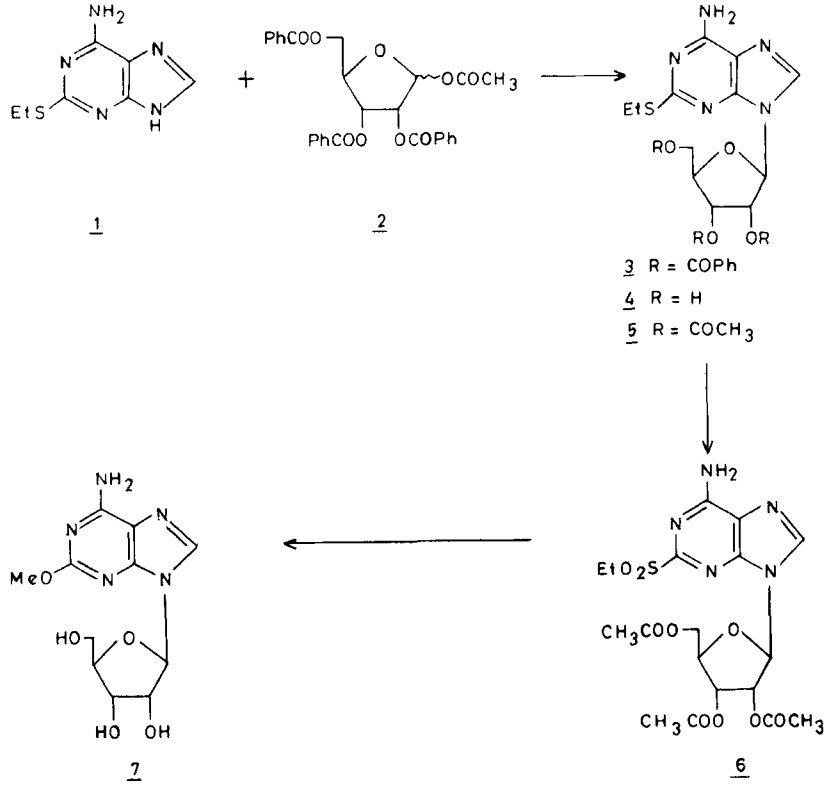
Introduction

Adenosine, a natural purine metabolite, is continuously produced in the body by many tissues including the vascular endothelium,[1] heart, brain, skeletal muscle[2], and blood platelets.[3] Adenosine is a potent vasodilator and contributes greatly in vascular blood flow

and oxygen supply.[2] In addition, adenosine is a potent antiplatelet agent.[4] Adenosine is rapidly metabolized in most tissues, primarily by adenosine deaminase and adenosine kinase[5,6], producing low levels of plasma adenosine (0.1 - 0.3 μM).[3,7] This laboratory has recently reported that plasma adenosine plays an important role in negative modulation of platelet thrombotic activity.[3] During the past decade, adenosine research has greatly contributed to our understanding of the physiological significance of this biological metabolite. A large number of adenosine analogs have been synthesized to identify biologically active agents.[8,9] In addition, several biologically active adenosine derivatives have been isolated from the marine organisms. These include, 2-methoxyadenosine (spongosine) from the Caribbean sponge, *Crytotethia crypta*,[10] 1-methylisoguanosine from the ocean sponge, *Tedania digitata*, and 5'-deoxy-5-iodotubercidin from red algae of the Australian ocean.[11] Although these compounds have been examined for biological activities (e.g. muscle relaxation, hypothermia, heart rate etc.), their effects on platelets have not been investigated. The present study examines the antiplatelet effects of spongosine and its analogs, 8-azaspongosine and 2-ethylthioadenosine, which have been synthesized in our laboratory by a simple method with good recoveries.

Chemistry

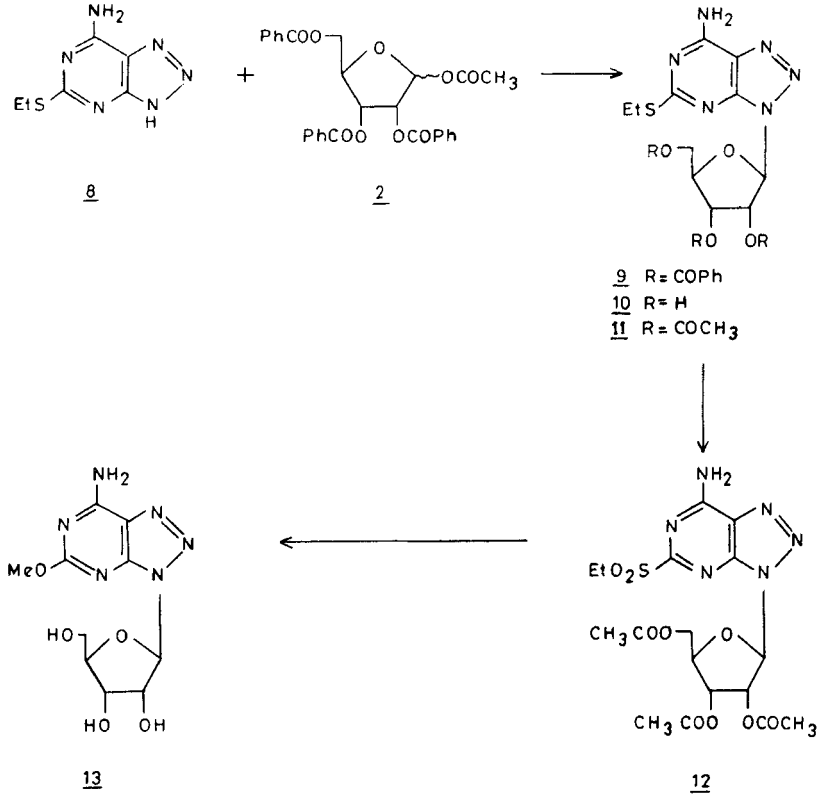
The processes hitherto known for the synthesis of 2-substituted adenosine analogs include: **a**. Condensation of a base and a ribose derivative,[12,13] and **b**. conversion from a naturally occuring nucleoside guanosine or 5-amino-1-ß-D-ribofuranosylimidazole-4-carboxamide (AICAR)[14,15]. Most such methods employ many steps consuming much longer time, or require expensive compounds. For example, condensation of 2,6-dichloropurine with an appropriate ribosyl derivative by Saneyoshi and Satoh[13] gave a good yield of the nucleoside, however, 2,6- dichloropurine is an expensive intermediate and is not readily prepared. The condensation of 2-alkylthioadenine with an appropriate ribose derivative in the presence of mercuric chloride gave inconsistent and low yields of the nucleoside[12]. The fusion of 2-methylthio-6-chloropurine with a ribose derivative was also incomplete and accompanied with much decomposition[12]. During the synthesis of 8-azaadenosine, Montgomery et al[16] condensed 5,7-bis(methylthio)triazolo[4,5-d]-pyrimidine with a less stable ribofuranosyl chloride and obtained an inseparable mixture



1 2 3 R = COPh 4 R = H 5 R = $COCH_3$ 7 6

SCHEME-1

of products. Here we report a facile synthesis of 2-ethylthioadenosine and 5-ethylthio-3-ß-D-ribofuranosyl-1,2,3-triazolo-[4,5-d]pyrimidine and their elaboration to spongosine (Scheme 1) and 8-azaspongosine (Scheme 2), respectively. Condensation of 2-ethylthioadenine (**1**)[17] with 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (**2**) in dry nitromethane in the presence of $SnCl_4$ gave 2-ethylthio-9-(2,3,5-tri-O-benzoyl-ß-D-ribofuranosyl)adenine (**3**) with 62% yield. Earlier, Saneyoshi and Satoh[13] employed these reactions conditions for the condensation of adenine with the ribose derivative (**2**) to obtain a good yield of the nucleoside. Deblocking of (**3**) with methanolic NH_3 at room temperature furnished 2-ethylthioadenosine (**4**). The UV absorption data of (**4**) (λ_{max} 275, 230 nm) suggests that the ribose moiety is attached at position 9 of the adenine[18]. The coupling constant of the anomeric proton (J=5 Hz) at 6.2 supports the ß-configuration of



SCHEME-2

the ribose. Before oxidation, compound (**4**) was protected by acetylation to afford (**5**). Using the method of Matsuda et al[19]., oxidation of (**5**) with $KMnO_4$ in acetic acid gave the corresponding sulfone (**6**). The reaction of compound (**6**) with NaOMe furnished spongosine (**7**).

For the preparation of 8-azaspongosine (**13**), 7-amino-5-ethylthio-1,2,3-triazolo[4,5-d]pyrimidine (**8**)[20] was condensed with (2) in the presence of $SnCl_4$ to give 7-amino-5-ethylthio-3-(2,3,5-tri-O-benzoyl-ß-D-ribo-furanosyl)-1,2,3-triazolo[4,5-d]pyrimidine (**9**) (60% yield). Deprotection gave 7-amino-5-ethylthio-3-ß-D-ribofuranosyl-1,2,3-triazolo-[4,5-d]pyrimidine (**10**) in 66% yield. The site of ribosylation was identified mainly from ^{13}C NMR data. The ^{13}C NMR chemical shift values of **10** and

7-amino-5-methylthio-3-ß-D-ribofuranosyl-1,2,3-triazolo[4,5-d]pyrimidine are identical. The UV absorption maxima of the nucleoside (**10**) (λ_{max} 247, 278 nm) also confirms ribosylation at N-3. Acetylation of (**10**) with acetic anhydride yielded 7-amino-5-ethylthio-3-(2,3,5-tri-O-acetyl-ß-D-ribofuranosyl)-1,2,3-triazolo[4,5-d]pyrimidine (**11**) which on oxidation gave the sulfonyl derivative (**12**). The downfield shift of methylene proton attached to sulfur from 3.1 to 3.7 in the PMR spectrum confirms the oxidation. The treatment of (**12**) with NaOMe in dry methanol yielded 7-amino-5-methoxy-3-ß-D-ribofuranosyl-1,2,3- triazolo-[4,5-d]pyrimidine (**13**).

Biological Evaluation

Platelet Aggregation: Informed consent was obtained from each subject before the blood was drawn. Blood was drawn from healthy donors who had not ingested drugs known to affect platelet functions for at least 8 days. Freshly drawn blood was anticoagulated with 0.1 volume of trisodium citrate (3.8%) and centrifuged at 277 g for 8 min to obtain PRP. The PRP was treated with saline (control) or adenosine deaminase (ADA, 2 units/ml). The control and ADA-treated PRP samples were then incubated with adenosine or an analog and after 5 min platelet aggregation was induced by collagen. Platelet aggregation in PRP was measured with the method of Born[21]. For evaluation of the antiaggregatory effects in whole blood, fresh human blood was anticoagulated with 0.1 volume of trisodium citrate (3.8% in water) and diluted to 30% hematocrit with NaCl (0.9%). Platelet aggregation was then measured by the impedance method of Cardinal and Flower[22]. The blood was incubated with various concentrations of adenosine or an analog for 5 min and platelet aggregation was induced by collagen. IC_{50} values were determined from the dose-response plots.

Results and Discussions

The antiplatelet effects of (**4**), (**7**) and (**13**) were compared with adenosine in presence and absence of ADA in human PRP. Platelet aggregation was induced by collagen. The data (Table I) show that in the absence of ADA, adenosine and the analogs (**4**) and (**7**) inhibit platelet aggregation. Analog (**4**) was more inhibitory (92%) than the analog (**7**) (67%), whereas, the aza-analog (**13**) is only weakly inhibitory (12%). In the presence of ADA (2 units/ml of PRP), adenosine and 8-azaspongosine (**13**) show no

TABLE I: Effects on platelet aggregation in human platelet-rich plasma

Agents	Concentrations (μM)	Inhibition (%) * Control	+ADA
Adenosine	10	46 ± 4	0 ± 0
Analog 4	100	92 ± 2	70 ± 23
Analog 7	100	67 ± 28	31 ± 3
Analog 13	100	12 ± 5	0 ± 0

Adenosine or an analog was incubated with normal PRP (Control) or PRP containing adenosine deaminase (+ADA) (2 units/ml) at 37° C. After incubation (5 min), platelet aggregation was induced by collagen, 1 μg/ml. *Data represent Mean ± SD from three separate experiments.

inhibitory effects on platelet aggregation suggesting that deaminated metabolites have no inhibitory effects. In contrast, the analogs (4) and (7) remain inhibitory although to a lesser extent, suggesting that these analogs are poor substrates for ADA.

The effects of adenosine and the analogs on collagen-induced platelet aggregation were also examined in human whole blood (Table II). The compounds (4) and (7) demonstrate strong inhibitory effects on platelet aggregation, whereas, adenosine (10 - 200 μM) shows no inhibition due to its rapid uptake and metabolism by blood cells. The IC_{50} values of 52 and 31 μM for (4) and (7) respectively were estimated from the dose-response plots, demonstrating that (4) is slightly more inhibitory than (7) in whole blood. However, in PRP where erythrocytes and leukocytes are absent, the analog (4) seems to have more inhibitory effects (Table I). This may be perhaps due to the differences in the cellular uptake and metabolism of these agents. The analog, 8-azaspongosine (10 - 200 μM) shows no inhibition at concentrations of platelet aggregation in whole blood.

Experimental

Melting points were determined in open capillaries. Proton magnetic resonanace (^{1}H NMR) spectra were obtained with a Perkin Elmer EM-60L and ^{13}C spectra on Brucker

TABLE II. Effects on platelet aggregation in human whole blood

Agents	IC_{50} (μM)
Adenosine	>200
Analog 4	52
Analog 7	31
Analog 13	>200

Human whole blood (Hct: 30%) was incubated with adenosine or adenosine analog (10 - 200 μM) at 37° C, and after 5 min platelet aggregation was induced by ADP (10 μM). The IC_{50} values were estimated from the dose-response plots.

WM 400. The chemical shift values are expressed in parts per million relative to tetramethylsilane as an internal standard. Ultraviolet (UV) spectra were recorded using Perkin-Elmer 202 spectrophotometer (λ_{max} in nm). Thin layer chromatography employed Silicagel F_{254} plates, and the spots were visualized under a UV lamp or after spraying with 1% H_2SO_4 in MeOH followed by heating. Evaporations were performed under reduced pressure below 40°C with a rotatory evaporator.

2-Ethylthio-9-(2,3,5-tri-O-benzoyl-ß-D-ribofuranosyl)adenine (3)

2-Ethylthioadenine (1), (1.0 g, 5.12 mmole) and 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (3.1 g, 6.15 mmole) were stirred in nitromethane at room temperature. The catalyst $SnCl_4$ (1.0 ml) was added to the reaction mixture that was then stirred for 18 h. The solvent was removed under reduced pressure, and the residue was poured slowly into $NaHCO_3$ solution. The reaction mixture was extracted with $CHCl_3$, washed with H_2O, dried (Na_2SO_4), and the product was purified on a silicagel column. Elution with $CHCl_3$:MeOH (98:2, v/v) gave (3) as a foam (2.7 g, 62.5% yield). The product was then used in the next step.

2-Ethylthio-9-ß-D-ribofuranosyladenine (4)

Compound (3) (3.0 g, 4.7 mmoles) and methanolic NH_3 (80 ml, MeOH saturated with NH_3 at 0°) was kept at room temperature for 24 h. The solvent and excess NH_3 were

removed under reduced pressure, and the product was chromatographed on a silicagel column. Elution with $CHCl_3$:MeOH (98:2,v/v) gave (**4**) (1.4 g, 90% yield), m.p. 207° [211- 212°][14], MS: 327 (M^+), UV λ_{max} at pH 1, 270 (9.6), 228 (21.2), at pH 7, 275 (10.5), 230 (25.8), PMR (DMSO-d_6): 8.6 (s, 1H, H-8), 7.65 (bs, 2H, NH_2), 6.2 (d, 1H, J=5 Hz, H-1'), 3.4 (q, 2H, J=6Hz, S-CH_2), 1.6 (t, 3H, J=6Hz, CH_3) and other sugar protons.

Anal. Calcd. for $C_{12}H_{17}N_5O_4S$: C, 44.0; H, 5.2; N, 21.4.

Found: C, 43.7; H, 5.0; N, 21.1.

2-Ethylthio-9-(2,3,5-tri-O-acetyl-ß-D-ribofuranosyladenine (5)

A mixture of 2-ethylthioadenosine (**4**) (1.0 g, 3 mmoles) anhydrous pyridine (10 ml), and acetic anhydride (5 ml) was stirred at room temperature for 20 h. The excess reagent was removed under reduced pressure. The residue was taken up in $CHCl_3$, washed with H_2O, and dried (Na_2SO_4). After removal of solvent the product was chromatographed on a silicagel column. Elution of the column with $CHCl_3$:MeOH (98:2, v/v) gave **5** as an oil (1.1 g, 80% yield). MS: 453(M^+); PMR ($CDCl_3$): 8.55 (s,1H,H-8), 7.65 (bs, 2H, NH_2), 6.15 (d,1H,J=5Hz,H-1'), 3.1 (q, 2H,J=6Hz, S-CH_2CH_3), 2.0 (3s, 9H, $OCOCH_3$), 1.3 (t, 3H,J=6Hz ,S-CH_2CH_3).

2-Ethylsulfonyl-9-(2,3,5-tri-O-acetyl-ß-D-ribofuranosyl)adenine (6)

A mixture of (**5**) (1.1 g, 2.42 mmole), aqueous acetic acid (50%, 70 ml), and $KMnO_4$ (1.75 g) was stirred at 0° for 3 h. Excess reagent was decomposed with H_2O_2 (32%), and the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ solution was washed with H_2O, $NaHCO_3$ solution, dried (Na_2SO_4) under reduced pressure, and the product was chromatographed on a silicagel column. Elution with $CHCl_3$:MeOH (98:2, v/v) gave (**6**) (0.65 g, 55% yield), m.p. 161-2° (methanol), MS:485 (M^+) PMR ($CDCl_3$): 8.05 (s, 1H, H-8), 6.6 (bs, 2H, NH_2), 6.15 (d,1H, J=5Hz,H-1'), 3.45 (q, 2H, J=6Hz, $SO_2CH_2CH_3$), 2.0 (bs, 9H, $OCOCH_3$), 1.35 (t, J=6Hz, CH_3) and other sugar protons.

Anal. Calcd. for $C_{18}H_{23}N_5O_9S$: C, 44.53; H, 4.77; N, 14.42.

Found: C, 44.20; H, 4.53; N, 14.0.

2-Methoxy-9-ß-D-ribofuranosyladenine (7)

Compound (6) (0.3 g, 0.7 mmole) in methanol (30 ml) was refluxed with freshly prepared NaOMe (0.65 g, Na in 12 ml abs. MeOH) for 2 h. The resulting mixture was cooled, neutralized with AcOH, evaporated under reduced pressure, and the residue was chromatographed on a silicagel column. Elution with $CHCl_3$:MeOH (90:10, v/v) gave the product (7) (0.16 g, 87% yield), m.p. 189-90° (Water) (190°)[24], MS: 297 (M^+), UV λ_{max} at pH 1, 270 (10.0), 215 (9.6) at pH 7, 266 (10.5), 215 (9.3); PMR(DMSO- d_6): 8.55 (s, 1H, H-8), 7.65 (bs, 2H, NH_2), 6.2 (d, 1H, J=6Hz, H-1'), 4.1 (s, 3H, OMe) and other sugar protons.

Anal. Calcd. for $C_{12}H_{17}N_5O_4S$: C, 44.0; H, 5.2; N, 21.4.

Found: C, 43.7; H, 5.0; N, 21.1.

7-Amino-5-ethylthio-3-(2,3,5-tri-O-benzoyl-ß-D-ribofuranosyl)-1,2,3-triazolo[4,5-d]-pyrimidine (9)

7-Amino-5-ethylthio-1,2,3-triazolo[4,5-d]pyrimidine (8)[20] (3.0 g, 15.3 mmole) and 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (9.0 g, 17.8 mmole) were stirred in acetonitrile at room temperature. $SnCl_4$ (3 ml) was added to the reaction mixture that was then stirred for 18 h. The solvent was removed under reduced pressure, and the residue was poured slowly into $NaHCO_3$ solution. The reaction mixture was extracted with $CHCl_3$, washed with H_2O, dried (Na_2SO_4) under reduced pressure, and the product was purified on a silicagel column. Elution with EtOAc:Hexane (70:30, v/v) afforded (9) as an oil (6 g, 60% yield). The product was then used in the next step.

7-Amino-5-ethylthio-3-ß-D-ribofuranosyl-1,2,3-triazolo[4,5-d]pyrimidine (10)

Compound (9) (3.0 g, 4.7 mmole) and methanolic NH_3 (150 ml) was kept at room temperature for 30 h. The methanol and excess NH_3 were removed under reduced pressure, and the product was chromatographed on a silicagel column. Elution of the column with EtOAc:hexane (70:30, v/v) afforded 10 (1.0 g, 66% yield), m.p. 178° (methanol) MS: 328 (M^+), IR (KBr): 3310-3420 (OH, NH_2); PMR (DMSO-d_6): 7.5 (bs, 2H, NH_2), 6.35 (d, 1H,J=4Hz, H-1'), 4.6 (m, 1H, H-4'), 4.25 (m, 1H, H-3'). 3.8 (m, 1H, H-2'), 3.2 (q, 2H, J=6Hz, SCH_2CH_3), 1.4 (t, 3H, J=6Hz, CH_3), ^{13}CMR (DMSO-d_6): 170 (C-5), 155.1 (C-7), 149.7 (C-3a), 122.7 (C-7a), 89.5 (C-1'), 85.9 (C- 4'), 72.6

(C-2'), 70.7 (C-3'), 61.9 (C-5'), 24.6 (-S-CH_2-CH_3), 14.5 (-S-CH_2CH_3), UV λ_{max} at pH 1, 246 (0.7), 280 (1.3), at pH 7, 247 (3.0), 278 (2.1).

Anal. Calcd. for $C_{11}H_{16}N_6O_4S$: C, 40.23; H, 4.9; N, 25.59.

Found: C, 40.2; H, 4.5; N, 26.0.

7-Amino-5-ethylthio-3-(2,3,5-tri-O-acetyl-ß-D-ribofuranosyl)-1,2,3-triazolo[4,5-d]-pyrimidine (11)

A mixture of (**10**) (1.0 g, 3.04 mmole), dry pyridine (30 ml), and acetic anhydride (1 ml) was stirred at room temperature for 20 h. The excess reagent was removed under reduced pressure. The residue was dissolved in $CHCl_3$, washed with H_2O, dried (Na_2SO_4) under reduced pressure, and the product was chromatographed on a silicagel column. Elution of the column with $CHCl_3$:MeOH (98:2,v/v) gave (**11**) as an oil (0.8 g, 60% yield), MS (m/z): 455 (M^+), PMR ($CDCl_3$): 7.5 (bs, 2H, NH_2), 6.35 (d, 1H, J=4Hz, H-1'), 4.2-4.7 (m, 3H, H-2', H-3', H-4'), 3.1 (q, 2H, -S-CH_2-CH_3), 2.05 (bs, 9H, $COCH_3$), 1.3 (t, 3H, CH_3).

7-Amino-5-ethylsulfonyl-3-(2,3,5-tri-O-acetyl-ß-D-ribofuranosyl)-1,2,3-triazolo-[4,5-d]-pyrimidine (12)

A mixture of (**11**) (0.8 g, 1.8 mmole), aqueous acetic acid (50%, 70 ml), and $KMnO_4$ (1.0 g) was stirred at O° for 3 h, H_2O_2 (32%, 25 ml) was then added to decompose excess reagent. The resulting mixture was extracted with $CHCl_3$, washed with H_2O, and dried (Na_2SO_4) under reduced pressure. The product was purified on a silicagel column using hexane:EtOAc (60:40, v/v) to give (**12**) as an oil (0.6 g, 55% yield), MS: 486 (M^+), PMR ($CDCl_3$): 1.4 (t, 2H, SO_2-CH_2-CH_3), 3.7 (q, 2H, $SO_2CH_2CH_3$), 6.45 (d, 1H, $C_{1'}$-H), 2.1 (bs, 9H, $COCH_3$) and other protons, UV λ_{max} at pH 1, 270 (5.78), 218 (4.89), at pH 7, 278 (5.90), 268 (5.92), 222 (5.0), 218 (5.0).

Anal. Calcd. for $C_{17}H_{22}N_6O_9S$: C, 41.97; H, 4.55; N,17.27.

Found: C, 42.0; H, 4.32; N, 17.53.

7-Amino-5-methoxy-3-ß-D-ribofuranosyl-1,2,3-triazolo[4,5-d]pyrimidine (13)

To compound (**12**) (1.0 g, 2 mmole) in absolute MeOH (100 ml) was added freshly prepared NaOMe (3.4 ml, 0.65 g Na in 12 ml absolute MeOH), and the mixture was

refluxed for 5-6 h. The resulting mixture was cooled, neutralized with acetic acid, evaporated under reduced pressure, and the product was chromatographed on a silicagel column. Elution of the column with EtOAc:MeOH (95:5, v/v) afforded (**13**) (0.4 g, 65.5% yield), m.p. 152-3° (methanol), MS: 298 (M^+), PMR (DMSO-d_6): 8.2 (s, 2H, NH_2), 6.1 (d, 1H, J=4Hz, H-1'), 4.2-4.8 (H-3',H-4', OH), 3.9 (s, 3H, OCH_3), 3.6 (m, 2H, H-5'), ^{13}CMR (DMSO-d_6): 164.8 (C-5), 157.0 (C-7), 151 (C-3a), 122.2 (C-7a), 89.4 (C-1'), 86.0 (C-4'), 72.6 (C-2'), 70.8 (C-3'), 62.0 (C-5'), 54.6 (OCH_3); UV λ_{max} at pH 1, 289 (6.7), 251 (5.2), at pH 7, 295 (6.6), 253 (5.4).

Anal. Calcd. for $C_{10}H_{14}N_6O_5$: C, 40.26; H, 4.73; N, 28.18.

Found: C, 40.01; H, 4.82; N, 27.85.

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