Facile Synthesis of 2-[(3-Aminopropyl)thio]adenosine 5′-Diphosphate: A Key Intermediate for the Synthesis of Molecular Probes of Adenosine 5′-Diphosphate Function†

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Adenosine 5′-diphosphate (ADP) and, uniquely, its C-2 derivatized analogues are able to induce platelet activation. We here report the synthesis of 2-[(3-aminopropyl)thio]-ADP from ADP itself via 1,N^6-etheno-ADP. 2-[(3-Aminopropyl)thio]-ADP induced platelet aggregation with a potency about one-seventh that of ADP itself and should prove a useful intermediate in the synthesis of other probes of platelet function.

Adenosine 5′-diphosphate interacts with a variety of enzyme systems as substrate, cofactor, or allosteric effector.[2] ADP can also function as an agonist in the activation of blood platelets, and thus it plays an important role in hemostasis and thrombosis[3] and, perhaps, in tumor metastasis.[4] Only ADP and those analogues containing a substitution for the C-2 hydrogen of the purine ring are effective in stimulating the platelet bioresponse: substitutions at N^1, N^6, C-8, the ribose sugar, or the pyrophosphate moieties yield compounds with little or no platelet aggregating activity.[5]

We here describe the facile synthesis of a versatile new intermediate, 2-[(3-aminopropyl)thio]-ADP from ADP itself under conditions sufficiently mild that little phosphate hydrolysis occurs and we describe its activity as a platelet aggregating agent. 2-[(3-Aminopropyl)thio]-ADP can be used to prepare a series of C-2 substituted derivatives as molecular probes by coupling additional groups to the end of the spacer arm via the primary amine; for example, (i) the succinimido esters of activated carboxylic acids such as p-azidobenzoic acid would yield photoaffinity reagents; (ii) coupling to activated Sepharose would produce matrixes for ADP affinity chromatography; (iii) a radiotracer with a high specific activity could be prepared by reaction with ^{125}I-labeled Bolton–Hunter reagent; and (iv) the attachment of biotin, when used in conjunction with appropriately labeled avidin derivatives, would afford a means of cytochemical visualization.

Results and Discussion

Chemistry. The merit of the synthetic route employed for the preparation of 2-[(3-aminopropyl)thio]-ADP (**6**, Scheme I) is centered around the use of the etheno group, which protects one portion of the molecule while activating another: removal of the 2-carbon is easier than in the compounds used by Meyer[6] and Kikugawa[7] as the source of their 2-nor intermediates and reinsertion of the 2-carbon to afford the 2-thio compound can be accomplished under much milder conditions so that the phosphodiester bonds are preserved. Finally, the etheno group enhances the reactivity of the sulfur, making it more susceptible to alkylation than is 2-thio-ADP, as already noted by Yamaji.[8]

ADP (**1**) was converted to the 1,N^6-etheno derivative **2** with chloroacetaldehyde.[9] HPLC detected the formation of an intermediate presumably similar to that detected by TLC in the analogous reactions with adenosine.[10] Treatment of 1,N^6-etheno-ADP with mild base afforded 3-β-D-ribofuranosyl-4-amino-5-imidazol-2-ylimidazole 5′-diphosphate (1,N^6-etheno-2-nor-ADP, **3**).[11,12] The ring-opening process proceeds via a transient intermediate[13] detectable by HPLC. The approximate kinetic data for this transformation fit the form of two sequential pseu-

Scheme I. Synthetic Route for the Preparation of 2-[(3-Aminopropyl)thio]-ADP (RPP = Ribosyl Pyrophosphate)

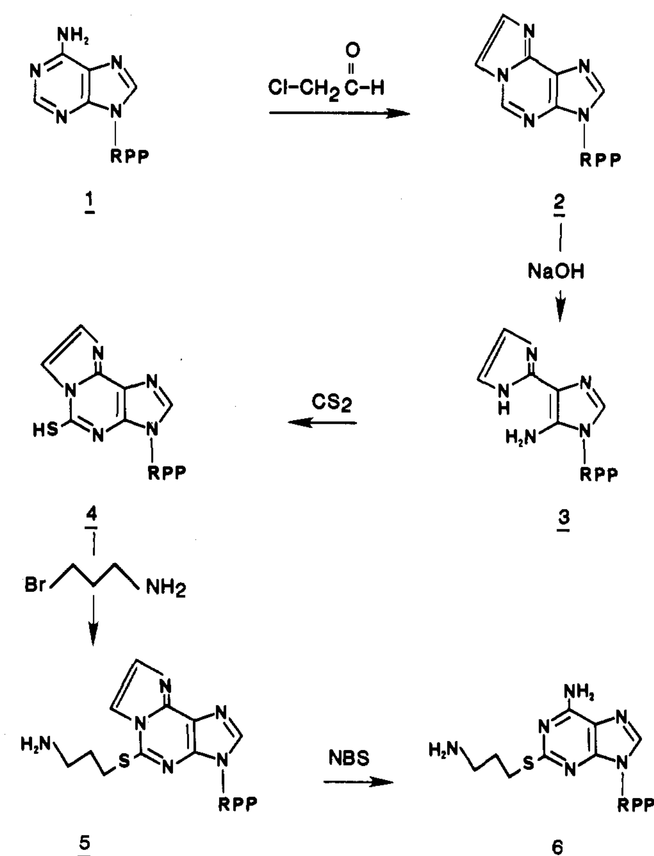

Table I. Kinetic Data for Ring-Opening Reaction of 1,N^6-Etheno-ADP

reaction	half-time,[a] min 0.1 M NaOH 25 °C	1.0 M NaOH 25 °C	1.0 M NaOH 37 °C
1,N^6-etheno-ADP → intermediate	32	4.5	1.4
intermediate → 1,N^6-etheno-2-nor-ADP	84	26	11

[a] Approximate half-times based on HPLC peak areas for the conversion 1,N^6-etheno-ADP (**2**) to 1,N^6-etheno-2-nor-ADP (**3**) through a transient intermediate. Aliquots of the reaction mixture were analyzed periodically by HPLC and the results subjected to computer modeling as described in the legend to Figure 1.

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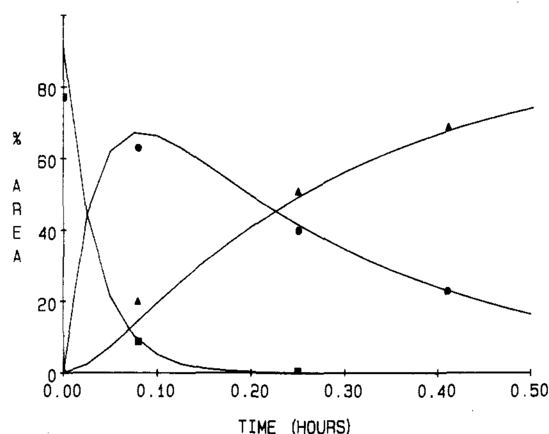

Figure 1. Kinetics of the reaction of 1,N^6-etheno-ADP with NaOH. Aliquots of the reaction mixture were analyzed periodically by HPLC and the relative peak areas of the starting material (1,N^6-etheno-ADP; ■), the intermediate (●) and the product (1,N^6-etheno-2-nor-ADP; ▲) plotted vs time. The curves were computer generated by fitting the experimental data to the rate equation for two sequential first-order reactions.[14]

do-first-order reactions[14] (Figure 1), both of which are accelerated by increasing the concentration of base (Table I).

We chose to reinsert 2-carbon into the 1,N^6-etheno-2-nor-ADP intermediate using carbon disulfide because of the reactivity of the product thiol. Yamaji and co-workers[15] prepared cyclic AMP derivatives with various substituents attached to the 2-position by thioether linkages, but their reaction conditions were not directly applicable to the ADP analogues. For example, in the ring-closure reaction, compound 3 was insoluble in *N*,*N*-dimethylformamide (DMF) so that the procedure had to be redesigned to accommodate the polarity and hydrophilicity of dimethyl sulfoxide (DMSO).

Alkylation of compound 4 was carried out with 3-bromopropylamine prior to removal of the etheno group to protect the sulfur from oxidation in the subsequent step by *N*-bromosuccinimide.[15] That alkylation of the sulfur

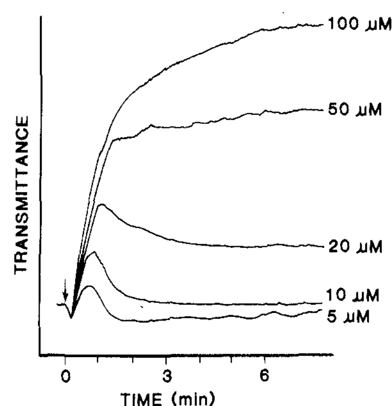

Figure 2. Aggregation of human blood platelets by 2-[(3-aminopropyl)thio]-ADP. To a stirred suspension of platelet-rich plasma (PRP, 490 mL) at 37 °C was added 10 µL of a solution of 2-[(3-aminopropyl)thio]-ADP (arrow), yielding the final concentrations indicated to the right of the ordinate. The change in light transmittance was recorded as a function of time.

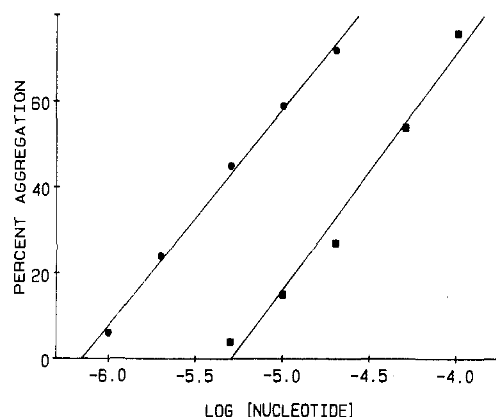

Figure 3. Comparison of the extent of platelet aggregation by ADP and by 2-[(3-aminopropyl)thio]-ADP. Platelet-aggregation experiments were performed as described and the height of the first wave produced by various concentrations of ADP (●), and by 2-[(3-aminopropyl)thio]-ADP (■) was determined. The extent of aggregation is plotted vs the log of the nucleotide concentration, and the lines shown were determined by the method of least squares.

had occurred was substantiated by the appearance of three new resonances in the alkyl region of the ^1H and ^{13}C NMR spectra of the nucleotide. When alkylation was attempted with 2-bromoethylamine, a mixture of products was detected possibly due to the formation of higher order homologues by intermolecular reactions involving three-membered cyclic aziridines.[16]

The steps needed to remove the etheno group and thus regenerate the intact purine ring system do not detract from the versatility of the synthetic scheme as they can also be accomplished specifically and under moderate conditions. The removal of the etheno group by reaction with *N*-bromosuccinimide has been described for some 2-substituted cyclic AMP analogues.[17] This reaction worked well with our 2-substituted ADP derivative, but substantial changes were required in the method of purification. ^1H and ^{13}C NMR spectra confirmed that removal of the two-carbon etheno group had occurred. The ^{13}C NMR spectrum is similar to that of ADP with the notable difference being additional resonances in the alkyl

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region, while the proton spectrum indicated a single aromatic proton corresponding to C-8, but with the three multiples in the alkyl region of the aminopropyl thioether group and no C-2 proton.

The product ADP analogue 2-[(3-aminopropyl)thio]-ADP was found to be an effective agonist for platelet aggregation (Figure 2), and this compound has similar effects to ADP itself. Thus at low cncentrations both ADP and the analogue give reversible aggregation and a biphasic response at higher concentrations. Further, the log plots of extent of aggregation versus concentration are parallel for the two compounds (Figure 3) except that a sevenfold higher concentration of the analogue is required.

Experimental Section

Chemistry. Proton and ^{13}C magnetic resonance (NMR) spectra were run on a JEOL FXQ-90 spectrometer with a tunable probe. Nucleotide samples were dissolved to a concentration of ca. 0.05 M in D_2O with 3-(trimethylsilyl)propionic acid as reference, and chemical shifts are reported in parts per million, relative to this internal standard. Some spectra were run without the internal standard to avoid the need for its subsequent removal. In such cases the shift values were calculated relative to this reference by assigning the resonance line due to HOD a value of 4.91 ppm in the proton NMR and assigning the midpoint of the pulse a value of 91 ppm in the case of ^{13}C NMR. Ultraviolet measurements were made on either a Gilford single-beam spectrophotometer or a Cary Model 15 double-beam spectrophotometer. Phosphate analysis[18] of the purified 2-substituted derivatives confirmed the presence of 2 mol of phosphate/mol of chromophore, and the phosphate values were also correlated with ultraviolet absorbance data to determine molar extinction coefficients. Elemental analysis, weight loss upon drying, and Karl Fisher determination of water were performed by Galbraith Laboratories, Knoxville, TN. Analytical results indicated by elemental symbols were within ±0.4% of the theoretical values. To improve HPLC resolution, the nucleotide sample were made weakly alkaline with dilute NaOH and immediately injected on an Altex Ultrasil SAX anion-exchange column at a concentration of 2–10 mM in a volume of 20 μL or less. The column was then eluted with a linear gradient of 10–700 mM NaH_2PO_4 over 10 min at 2 mL/min and nucleotides were detected at 254 nm in an optical flow cell.

1,*N*^{6}-Etheno-ADP (2). This compound was prepared by minor modification of the method of Secrist et al.[9] The product nucleotide was precipitated as a gummy solid by the addition of absolute ethanol, converted to a white powder by triturating with a glass rod, filtered off, and washed with absolute ethanol. The product (yield ~90%) was 85–90% pure by HPLC (t_R = 8.8 min) and by colorimetric phosphate analysis. The UV and ^{1}H NMR spectra of this sample were similar to those for 1,*N*^{6}-ethenoadenosine,[19] and the product was not further purified.

1,*N*^{6}-Etheno-2-nor-ADP (3). This compound was prepared by base hydrolysis of compound **2** (1 g, 1.5 mmol) as described for the synthesis of 1,*N*^{6}-etheno-2-noradenosine[11] but with ~1 M NaOH for 3 h at 37 °C to increase the rate of reaction. The basic solution was then chilled to 4 °C and applied directly to a column (1.5 cm × 30 cm) of cation-exchange resin (AG-50 × 8, H^+), equilibrated at 4 °C.[20] 1,*N*^{6}-Etheno-ADP and any unreacted ADP from the previous step passed directly through the column along with an unidentified brown impurity. The major UV-absorbing peak, which eluted after the passage of 5 column volumes of water, was pooled and concentrated to dryness. Compound **3** (yield 80%) was 98+% pure by HPLC (t_R = 6.7 min) and phosphate analysis. UV and ^{1}H NMR spectra were similar to those previously reported.[13] The fractions eluting immediately after the most concentrated product fractions were light green due to an unidentified impurity and were discarded, but the green color served as a good indicator of the progress of chromatography.

1,*N*^{6}-Etheno-2-mercapto-ADP (4). All procedures involving the preparation and purification of compound 4 were carried out in the fume hood. To 100 mL of dry DMSO in a glass-stoppered Erlenmeyer flask equipped with a magnetic stirrer were added compound **3** (1 g, 2.3 mmol), carbon disulfide (10 mL, 166 mmol), and anhydrous Li_2CO_3 (1 g, 13.5 mmol). The progress of the ring-closure reaction was followed by HPLC and UV spectroscopy. After stirring for 7 days at room temperature, which afforded the maximum yield, the mixture was filtered through glass fiber paper with a Buchner funnel and the hygroscopic solid quickly washed with 2 × 5 mL of DMSO. The combined filtrates were treated with 1 volume of hexane and 2 volumes of methylene chloride to precipitate the product nucleotide, and it was found convenient to refrigerate this mixture at 4 °C overnight to complete the precipitation. The precipitate was then separated by centrifugation for 15 min at 10 000 rpm in solvent-resistant bottles, and the solid was washed by resuspension in 2 × 10 mL of methylene chloride and recentrifuged. The organic solvent remaining after decantation was removed under reduced pressure with a vacuum pump and cold trap. This crude precipitate was very hygroscopic and drying was effected while the solid was still in the centrifuge bottles. The material thus obtained (ca. 1.2 g) was contaminated with various sulfur residues, being only about 60% pure as determined by HPLC.

To effect further purification, a sample (1 g) was dissolved in 50 mL of H_2O and made weakly alkaline with dilute NaOH, applied to a column (1.5 cm × 30 cm) of cation-exchange resin (AG-50 × 8, H^+) at room temperature, and eluted with water. The first fractions off the column were yellowish and were discarded, while the purified product was detected by UV absorbance in the clear fractions immediately following, which were pooled and concentrated to dryness to yield compound 4 (typically 0.5 g, 1 mmol, 50% yield[21] based on compound **3**), which was determined to be 97+% pure by HPLC (t_R = 8.2 min): ^{1}H NMR δ 8.39 (s, 1), 8.17 (d, 1, J = 1.5 Hz), 7.48 (d, 1, J = 1.5 Hz), 6.30 (d, 1, J = 6 Hz), 4.3–5.0 (m, 5); ^{13}C NMR (aromatic region) δ 167, 147, 142, 138, 121, 119, 115 (sugar region) 91, 86 (d), 77, 73, 68 (d); UV λ_{max} nm ($\epsilon \times 10^{-3}$) pH 1, 310.5 (17.0) 239 (13.4); pH 11, 318 (12.3) sh 292 (8.9) sh 280 (6.1), 248 (11.1). *Note of caution*: A number of the later fractions following elution of the product were noticeably yellow and had a strong pungent odor. These fractions should be discarded carefully to avoid exposure to this stench.

1,*N*^{6}-Etheno-2-[(3-aminopropyl)thio]-ADP (5). Compound **4** (1 g, 2.0 mmol) was dissolved in DMF (50 mL) and triethylamine (1 mL, 10 mmol) in a glass stoppered Erlenmeyer flask followed by the addition of bromopropylamine hydrobromide (1 g, 4.5 mmol) dissolved in a small volume of DMF (3 mL). After 20 min a precipitate began to form, and the reaction was allowed to continue for 3 h. The off-white solid was collected by centrifugation and washed with abolute ethanol (3 × 5 mL) by centrifugation and decantation and finally dried under reduced pressure (1.3 g, 1.7 mmol). For further purification 1 g of the crude solid was dissolved in 15 mL of distilled water, the pH was adjusted to 4.0 with 1 M HCl, and the solution was applied to a column (1.5 cm × 30 cm) of cation-exchange resin (AG-50 × 8, H^+) equilibrated with 10 mM acetic acid. After the column was washed with 1 column volume of 10 mM acetic acid and an additional column volume of 1 M LiCl in 10 mM acetic acid, the product was eluted with a 500-mL linear gradient of 1–5 M LiCl in 10 mM acetic acid. UV-absorbing fractions eluting at a LiCl concentration

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(20) The temperature at which this chromatographic step is carried out is crucial to obtaining a good yield; when the same procedure is carried out at room temperature (instead of 4 °C), up to 35% of the product can be lost due to hydrolysis to 1,*N*^{6}-etheno-2-nor-AMP, which was eluted from the column with 10% ammonium acetate and characterized by UV and phosphate analysis. Presumably, the lower temperature reduces the effect of the heat of neutralization on the column and thus reduces the loss due to hydrolysis to an acceptable 7% overall.

(21) It was observed that although the reaction reached equilibrium in 7 days, the extent of conversion varied between 60% and 85% and appeared to be dependent on an undetermined variable such as water in the system. Most of the remaining nucleotide could be accounted for as unreacted compound **3**, which eluted from the column with ca. 5 column volumes of water and could be recycled.

of 2 M were concentrated to a slurry by rotary evaporation, and the product was precipitated with cold ethanol (20 mL) and washed on a sintered glass funnel with cold absolute ethanol (3 × 10 mL) to remove residual LiCl. Drying in vacuo over P_2O_5 yielded compound **5** (1.0 mmol, 0.63 g, 65% yield based on compound 4), which was 98% homogeneous as determined by HPLC (t_R = 8.8 min): ^{1}H NMR δ 8.63 (s, 1), 7.76 (s, 2), 6.25 (s, 1), 4.3–5.0 (m, 5), 3.64 (m, 2), 3.28 (m, 2), 2.31 (m, 2); ^{13}C NMR (aromatic region) δ 151, 144.5, 143.9, 140, 128, 119, 115 (sugar region), 91, 86(d), 77, 73, 68 (alkyl region), 41, 31, 29; UV λ_{max} nm ($\epsilon \times 10^{-3}$) pH 1, 293 (16.7), 234 (22.5); pH 11, 304 (9.9), 282 (10.1), sh 273 (8.32). Anal.[22] ($C_{15}H_{22}N_6P_2O_{10}S$) C, P; H: calcd, 3.86; found, 4.63; N: calcd, 14.74; found, 14.19. This compound appears to be susceptible to atmospheric oxidation, possibly to a sulfoxide. Thin-layer chromatography on silica in isobutyric acid (70%)/water (25%)/ammonium hydroxide (5%) gave two spots when run in one dimension and each spot could be resolved further into two spots when run with the same buffer in the second dimension, indicating that the second spot was formed during the chromatography: this oxidation was avoided by carrying out TLC in a N_2 atmosphere.

2-[(3-Aminopropyl)thio]-ADP (6). The etheno group was removed from compound **5** (0.95 g, 1.5 mmol) with *N*-bromosuccinimide (350 mg, 2 mmol) in a manner similar to that reported by Yamaji.[15] The solution was stirred at 25 °C while the pH was maintained at 3.0 by periodic addition of 1 M NaOH. After 2 h the pH was raised to 12 with NaOH and the solution stirred for an additional hour. The pH of the solution was then lowered to 3 with 6 M HCl and added to a column (1.5 × 30 cm) of cation-exchange resin (AG-50 × 8, H^+) that had been equilibrated with 10 mM acetic acid. After the column was washed with 1 column volume of 10 mM acetic acid, the product was eluted with a 600-mL linear gradient of 0–2.5 M LiCl containing 10 mM acetic acid. The UV-absorbing fractions eluting at 1 M LiCl were concentrated to a slurry by rotary evaporation and cold ethanol (20 mL) was added to yield the nucleotide product (ca. 200 mg) that was homogeneous as judged by HPLC (>95%) but was slightly yellow.

A further purification was effected by dissolving this solid (100 mg) in water (5 mL) and applying it to a column (1.5 cm × 18 cm) of anion-exchange resin (DEAE-cellulose) equilibrated with distilled water. The column was washed with 1 column volume of water and eluted with a 600-mL linear gradient of 0–0.1 M LiCl. UV-absorbing fractions eluting at 0.04 M LiCl were combined and concentrated to a slurry. The product was precipitated with cold ethanol (20 mL), collected on a sintered glass funnel, washed with cold absolute ethanol (3 × 10 mL) to remove residual LiCl, and dried in vacuo over P_2O_5. This procedure yielded compound **6** (0.4 mmol, 24% yield based on compound **5** by UV analysis), which was 98% homogeneous by HPLC (t_R = 6.5 min): ^{1}H NMR δ 8.48 (s, 1), 6.24 (d, 1, *J* = 6 Hz), 4.3–5.0 (m, 5), 3.42 (m, 2), 3.19 (m, 2), 2.21 (m, 2); ^{13}C NMR (aromatic region) δ 167, 158, 153, 142, 119 (sugar region) 91, 86 (d), 76, 73, 67 (d) (alkyl region) 41, 30 (2× intensity); UV λ_{max} nm ($\epsilon \times 10^{-3}$) pH 1, 268 (14.7); pH 11, 274 (15). Anal.[22] ($C_{13}H_{22}N_6P_2O_{10}S$) C, N, P; H: calcd, 4.02; found, 4.85.

Biochemistry. Whole blood, collected into $^1/_{10}$ volume of acid/citrate/dextrose buffer (5.5 mM dextrose, 128 mM NaCl, 4.26 mM NaH_2PO_4, 7.46 mM Na_2HPO_4, 4.77 mM Na_3·citrate, 2.35 mM citric acid), was used to prepare platelet-rich plasma (PRP) by centrifugation (3 min × 3000*g*). Aggregations were performed in a Payton Dual Channel aggregometer, using PRP (490 μL, containing (3–5) × 10^8 platelets/mL), which was stirred at 37 °C in a glass cuvette. After sufficient time for temperature equilibration, the sample of PRP was challenged with varying amounts of nucleotide dissolved in 10 μL of water. Platelet-poor plasma (PPP) was used as the optical standard for complete aggregation and was prepared by centrifugation of PRP (3 min × 10000*g*). The change in light transmission upon platelet activation was recorded and the extent of maximum change in light transmittance for each addition of nucleotide was compared.

Registry No. 1, 58-64-0; 2, 38806-39-2; 3, 50663-84-8; 4, 110224-43-6; 5, 110224-44-7; 6, 110224-45-8; $ClCH_2CHO$, 107-20-0; $Br(CH_2)_3NH_2$·HBr, 5003-71-4.

(22) The percent compositions are calculated as the dilithium monohydrates. The amount of water of hydration was substantiated by ^{1}H NMR and was consistent with the difference found between the amount of weight loss upon drying and the total water content determined by the Karl Fisher method.

Nitrogen-Bridged Conformationally Constrained Etorphine Analogues. Synthesis and Biological Evaluation

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Three N–C8-bridged analogues **4–6** of the opiate etorphine (**3**) were synthesized and evaluated for opiate agonism and antagonism. In each case ring closure was effected by intramolecular N-alkylation with a suitably developed C8 side chain. Another key synthetic step was the selective monoprotection of diol 11, which allowed independent elaborations of the C7 and C8 side chains. All three analogues showed distinctly diminished agonist activities when compared to the corresponding *N*-methyl compound, 19(*R*)-*n*-butylorvinol (**3**). Furthermore, no antagonist activity was detectable. The results demonstrate that the conformation at the amino nitrogen in rigid morphinans is critical for potent opiate activity.

The necessity of a basic amino group in a molecule for the expression of opiate activity may be regarded as established fact and is undoubtedly the most clear-cut aspect of structure–activity relationships associated with the opiates. Yet, despite a vast amount of research and more than a few theories on the subject, the precise role of the amino group remains elusive. This is not surprising, considering the inherent difficulties associated with a direct study of the opiate–receptor complex. Recently, we focused our attention on two aspects of this problem, namely, the geometric orientation of the nitrogen lone pair of electrons in the active conformation of rigid morphinan opiates and the possibility of a conformational inversion being a determinant in agonist/antagonist differentiation.

An evaluation of the literature indicates that these topics have been previously addressed. For example, it has been shown that *D*-norlevorphanol (**1**), an inactive analogue of the opiate levorphanol (**2**), has its *N*-methyl group inverted with respect to that of levorphanol in the solid, as shown by the X-ray crystal structures of the hydrobromide salts.[1,2]

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