483.

12. Parson, W. B., Jr., *Am. J. Clin. Nutrition,* 1960, v8, 471.

13. Miller, O. N., Hamilton, J. G., Goldsmith, G. A., *Circulation,* 1958, v18, 489.

14. ———. *Scope,* Nov. 11, 1959.

15. Ratti, G., DeFina, E., *Lancet,* 1959, v11, 917.

16. Biggs, M. W., Friedman, M., Byers, S. O., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 641.

17. Chaikoff, I. L., Bloom, B., Siperstein, M. D., Kiyasu, J. Y., Reinhardt, W. O., Dauben, W. G., Eastham, J. F., *J. Biol. Chem.,* 1952, v194, 407.

18. Bollman, J. L., Cain, J. C., Grindlay, J. H., *J. Lab. and Clin. Med.,* 1948, v33, 1349.

19. Vahouny, G. V., Fawal, I., Treadwell, C. R., *Am. J. Physiol.,* 1957, v188, 342.

20. Vahouny, G. V., Woo, C. H., Treadwell, C. R., *ibid.,* 1958, v193, 41.

21. Sperry, W. M., Webb, M. J., *J. Biol. Chem.,* 1950, v187, 97.

22. Swell, L., Trout, E. C., Jr., Hopper, J. R., Field, H., Jr., Treadwell, C. R., *ibid.,* 1958, v230, 631.

23. Dixon, W. J., Massey, F. J., Jr., *Introduction to Statistical Analysis,* 2nd Ed., p121.

24. Beher, W. T., Baker, G. D., Anthony, W. L., Beher, M. E., *Henry Ford Hosp. Med. Bull.,* 1961, v9, 201.

25. Treadwell, C. R., Swell, L., Vahouny, G. V., Field, H., Jr., *J. Am. Oil Chem. Soc.,* 1959, v36, 107.

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## Metabolism of Chlorpromazine. IV. Identification of 7-Hydroxychlorpromazine and Its Sulfoxide and Desmethyl Derivatives.* (28088)

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Urinary excretion of unidentified glucuronic acid conjugates of chlorpromazine has been reported by many workers(1-5). Lin *et al.*(2) isolated 4 such conjugates from human urine by column and paper chromatography. Posner(3) found 3 phenolic metabolites which he suspected were the 3-hydroxy or 3,7-dihydroxy derivatives and/or their sulfoxides. Huang and Kurland(5) briefly reported isolation of 6 chlorpromazine glucuronides and 2 hydroxychlorpromazines, but it is not clear whether these compounds were ever identified, and if so, by what criteria. According to Forrest and Piette(6), "After 10 years of intensive clinical use of . . . chlorpromazine, the major urinary drug metabolites of the phenolic, polar fraction, have not been unequivocally identified, despite the fact that this drug was the subject of many metabolic investigations in numerous laboratories, and more data on its metabolic fate and type and amount of oxidative derivatives are available than for any other phenothiazine compound."

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Our earlier reports(7,4,8) were primarily concerned with the nonpolar metabolites of chlorpromazine. We now turn to its phenolic metabolites. As shown below, 7-hydroxychlorpromazine and 3 closely related derivatives have been identified in both human and dog urine by chemical and paper chromatographic methods.

*Experimental.* Human subjects included in this study were patients at the Hillside Hospital who were on a daily oral regimen of 300-1200 mg of chlorpromazine HCl. For the animal studies, female mongrel dogs used were fed chlorpromazine HCl or promazine HCl, 4 mg/kg b.i.d. Urine collections were made in the preliminary control period and again during medication.

*Isolation of metabolites.* Unconjugated metabolites of chlorpromazine (Fraction AB) were extracted from urine at pH 9 using 3 volumes of ethylene dichloride (Eastman). The organic extract was washed with dilute ammonia buffer, pH 9, dried over anhydrous sodium sulfate and taken to dryness. The residue was dissolved in methanol and fractionated by paper chromatography. The residual urine containing the glucuronide conju-

gates was incubated with one-tenth volume of β-glucuronidase (Ketodase, 5,000 units/ml) at pH 4.5 and 37°C for 18 hours. After adjustment to pH 9, the liberated phenols (Fraction C) were extracted with ethylene dichloride and worked up as described above.

*Chromatography.* The chromatographic solvent systems employed herein are indicated in Table II (footnote), together with their abbreviations. Further details on solvents and color reagents are given elsewhere(7). Whatman No. 3MM paper was used for the AmMe and KCl systems, Whatman No. 1 paper for the others. The paper used for the AmMe system was previously dipped in acetone containing 10% peanut oil. When spots were to be chromatographed in a second dimension they were eluted with methanol and respotted, or cut out and woven onto fresh chromatography paper. The pre- and post-Ketodase fractions (Fractions AB and C) were fractionated by 2-dimensional chromatography based on the systems EdBzF and BuEt.

*Interconversion of sulfoxides and sulfides.* The reduction of sulfoxides to sulfides was accomplished by refluxing for 30 minutes with zinc (300 mg) in glacial acetic acid (3 ml). The solution was then cooled and spotted directly on paper for chromatography. To negotiate the reverse reaction, the sulfide was oxidized on filter paper with 3% hydrogen peroxide (90% alcohol). After standing for several hours at room temperature, the sulfoxide formed was eluted with methanol and chromatographed.

*Methylation reactions.* O-methylation of the hydroxychlorpromazines was effected with diazomethane, $CH_2N_2$(9). The diazomethane was freshly prepared in ethereal solution from Diazald†(10) and chilled. A portion (2 ml) was mixed with a cold methanolic solution (2 ml) of the phenol and placed overnight in the refrigerator. The solvent was then removed under vacuum and the residue analyzed by chromatography.

A standard procedure for methylation of primary and secondary amines(11) was employed to N-methylate the desmethyl phenols. The desmethyl compound was mixed with methyl iodide and aqueous sodium carbonate and refluxed for 6 hours in a water bath at 82°C. The aqueous layer was adjusted to pH 9, and the methylated amine extracted with ethylene dichloride. The extract was dried over sodium sulfate and prepared for chromatographic analysis in the usual manner.

† Aldrich Chemical Co. trade name for N-methyl-N-nitroso-p-toluenesulfonamide.

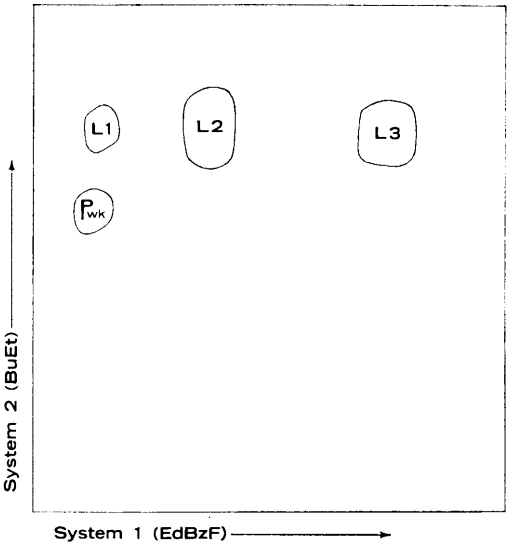


FIG. 1. Chromatographic separation of 7-hydroxychlorpromazine (L3) and its sulfoxide (Pwk) and desmethyl derivatives (L1 and L2) following their isolation from dog urine.

*Results. Isolation of four hydroxychlorpromazine metabolites.* A family of closely related phenols was isolated from the glucuronide hydrolyzate (Fraction C) of dog urine by chromatographing with EdBzF for 3-4 days. The faster-moving compounds were permitted to pass off the paper, leaving behind material which appeared as 3 discrete spots when stained with the persulfate reagent. The first was further resolved into 2 components with BuEt (Fig. 1). Three of the 4 spots seen in the Figure yielded a lavender persulfate color and are designated L1, L2 and L3. The remaining one, Pwk, gave a periwinkle (purple-blue) color.

The 3 lavender phenols were eluted from an unstained chromatogram for ultraviolet studies. They yielded absorption spectra similar to that of chlorpromazine, with a

shoulder at 280-285 mμ which is suggestive of a ring hydroxyl function, according to previous workers(2). The periwinkle phenol gave a sulfoxide spectrum. To determine the state of methylation of the 4 metabolites, their chromatograms were stained with the ninhydrin (primary amine) and nitroprusside (secondary amine) reagents(7). L1 reacted as a primary amine, L2 as a secondary amine, and L3 and Pwk as tertiary amines. Chromatographic analysis of the pre-Ketodase fraction (Fraction AB) revealed the presence of all 4 phenols (L1, L2, L3 and Pwk). This indicated that the metabolites appear in dog urine in both the conjugated (glucuronide) and free forms. In addition, both fractions were found to contain hydroxy derivatives which moved rapidly in the BzA and EdBzF systems. Analysis of the 2 metabolite fractions from human urine showed the presence of L1, L2, L3 and Pwk as well as several phenols not found in dog urine.

The urine of promazine-medicated dogs was also extracted and worked up as above. Four hydroxypromazine derivatives were isolated whose UV spectra, color reactions, and chromatographic behavior were analogous to those of the L1, L2, L3 and Pwk metabolites of chlorpromazine.

*Identification of Pwk as L3 sulfoxide.* Pwk was unlike the lavender phenols with respect to both its persulfate color reaction and oxidation state. Consequently it seemed that the 2 factors might be related. To test this hypothesis, the Pwk phenol was eluted from a chromatogram and reduced with zinc and acetic acid.‡ The product formed gave a lavender color with persulfate. It was further noted that the reduction product was chromatographically identical to L3 (Table I) and could not be differentiated by any known criteria. Conversely, L3 was readily oxidized by peroxide to yield a sulfoxide which gave a periwinkle color with persulfate, and was chromatographically indistinguishable from the Pwk phenol (Table I). Additional evidence for the relationship between L3 and Pwk is presented below.

‡ This method is routinely employed for conversion of sulfoxides to the sulfide stage. Sulfones are not reduced by the reagents(11, p578).

TABLE I. Interconversion of Pwk and L3 Metabolites.

| Solvent system† | $R_f$* L3‡ | Reduced Pwk‡ | Pwk§ | Oxidized L3§ |
|---|---|---|---|---|
| BzA | .23 | .23 | .045 | .046 |
| BuEt | .79 | .79 | .63 | .63 |
| IAmEtF | .83 | .83 | .56 | .56 |
| EdBzF | (20 cm)‖ | (20 cm)‖ | (1.5 cm)‖ | (1.5 cm)‖ |

* cm are given when solvent was permitted to drip off the paper.

† BzA = benzene-acetic acid-water (2:2:1), descending. BuEt=n-butanol-ethanol-water (15:2:5), ascending. IAmEtF=isoamyl alcohol-water-ethanol-formic acid (20:20:3:2), descending. EdBzF = ethylene dichloride-benzene-formic acid-water (3:1:4:2), descending.

‡ Yields a chlorpromazine-like UV absorption spectrum and a lavender persulfate color reaction.

§ Yields a sulfoxide UV absorption spectrum and a periwinkle persulfate color reaction.

‖ Distance travelled from origin in 72 hr.

The question may be raised as to whether the Pwk phenol is an N-oxide sulfoxide. We have observed that synthetic chlorpromazine-N-oxide sulfoxide(12) is not extracted into ethylene dichloride from alkaline medium. Since the hydroxy derivatives of this compound are still more polar in nature, they too would not be extracted into ethylene dichloride, and consequently would not appear on the chromatograms.

*Identification of L3 and Pwk.* The color reactions and solubility properties of L3 suggested that it was a simple monohydroxychlorpromazine. Unfortunately, synthetic hydroxychlorpromazine derivatives were not available to carry out comparison studies. The 2- and 4-isomers were tentatively ruled out when the promazine analogue of L3 was found to differ in $R_f$ and persulfate reaction (lavender) from 2-hydroxypromazine (orchid) and 4-hydroxypromazine (peach). The 3-hydroxy and 7-hydroxy isomers seemed likely possibilities, since the prototype of chlorpromazine (phenothiazine) is hydroxylated by animals at the 3- and 7-positions (13). The problem was resolved when we were provided with authentic 7-methoxychlorpromazine and 3-methoxychlorpromazine. Upon O-methylating the L3 metabolite with diazomethane (see *Experimental*), a de-

TABLE II. Chromatographic Identification of 7-Hydroxychlorpromazine (L3) and Its Sulfoxide (Pwk) in Urine.

| | $R_f$ * | | | | |
|---|---|---|---|---|---|
| | | | Oxidation product of | | |
| Solvent system† | O-methylated L3 | 7-Methoxy CP st'd | O-methylated L3 | 7-Methoxy CP st'd | O-methylated Pwk |
| 20% KCl | (13.5 cm)‡ | (13.5 cm)‡ | | | |
| AmMe | ( 4.5 " )‡ | ( 4.5 " )‡ | .72 | .72 | .72 |
| BzA | .76 | .76 | .24 | .24 | .24 |
| BuEt | .82 | .82 | .69 | .69 | .69 |
| IAmEtF | .86 | .86 | .61 | .61 | .61 |
| BuA | .80 | .80 | | | |
| IPrAm | .83 | .83 | | | |
| EdBzF | § | § | (8.5 cm)‖ | (8.3 cm)‖ | (8.5 cm)‖ |

* cm are given when solvent was permitted to run off the paper.

† 20% KCl = aqueous solution, descending. AmMe = ammonia (58%)-methanol (1:1), descending. BzA = benzene-acetic acid-water (2:2:1), descending. BuEt = n-butanol-ethanol-water (15:2:5), ascending. IAmEtF = isoamyl alcohol-water-ethanol-formic acid (20:20:3:2), descending. BuA = n-butanol-acetic acid-water (12:3:5), ascending. IPrAm = isopropanol-ammonia (58%)-water (8:1:1), ascending. EdBzF = ethylene dichloride-benzene-formic acid-water (3:1:4:2), descending.

‡ Distance travelled from origin in 15.5 hr.

§ Substance ran off paper before 24 hr.

‖ Distance travelled from origin in 24 hr.

rivative was obtained which yielded a lavender persulfate color similar to the 7-methoxy standard, and unlike the 3-isomer (sky blue). The methyl ether of L3 had the same $R_f$ as 7-methoxychlorpromazine in 7 solvent systems (Table II). When both compounds were oxidized to their sulfoxides with dilute alcoholic peroxide, they again showed similar chromatographic behavior (Table II), and both sulfoxides now yielded a periwinkle persulfate test. These data confirm the identification of L3 as 7-hydroxychlorpromazine. It also follows that Pwk is 7-hydroxychlorpromazine sulfoxide. This was confirmed by the chromatographic behavior of O-methylated Pwk (Table II) and its persulfate reaction (periwinkle). The structures and reactions of L3 and Pwk are given in Fig. 2.

*Identification of L1 and L2.* As indicated in the second paragraph under *Results,* the L1 phenol reacted as a primary amine, and L2 as a secondary amine. The color reactions and chromatographic behavior of these metabolites suggested that they were the desmethyl derivatives of 7-hydroxychlorpromazine (L3). This was verified by subjecting L1 and L2 to the routine treatment for N-methylation of primary and secondary amines (see *Experimental*). Both compounds were converted by methyl iodide to a common end-product which gave negative primary and secondary amine tests, and proved to be chromatographically indistinguishable from L3 (7-hydroxychlorpromazine) in 3 critical solvent systems (EdBzF, BzA, BuEt). The structures of L1 and L2 are given in Fig. 2.

*Discussion.* Retrospective studies indicate that 3 of the 7-hydroxychlorpromazines identified here were first detected as trace components of the nonpolar metabolite fraction in our earlier work(4,7). Spot no. 1 referred to previously now appears to have been a mixture of 7-hydroxychlorpromazine sulfoxide and 7-hydroxy-nor$_2$chlorpromazine, while spot no. 9 was probably 7-hydroxy-nor$_1$chlorpromazine contaminated with a sulfoxide. Incidentally, 7-hydroxychlorpromazine also appears in the nonpolar metabolite chromatogram if sufficient urine extract is spotted out.

The four 7-hydroxychlorpromazines (Fig. 2) actually constitute 8 metabolites in urine, since they are excreted as the free hydroxy compounds as well as their glucuronides. These compounds when combined with the 5 nonphenolic metabolites present in the nonpolar fraction (ref. 8, Fig. 1) add up to 13. Additional hydroxy derivatives are present in

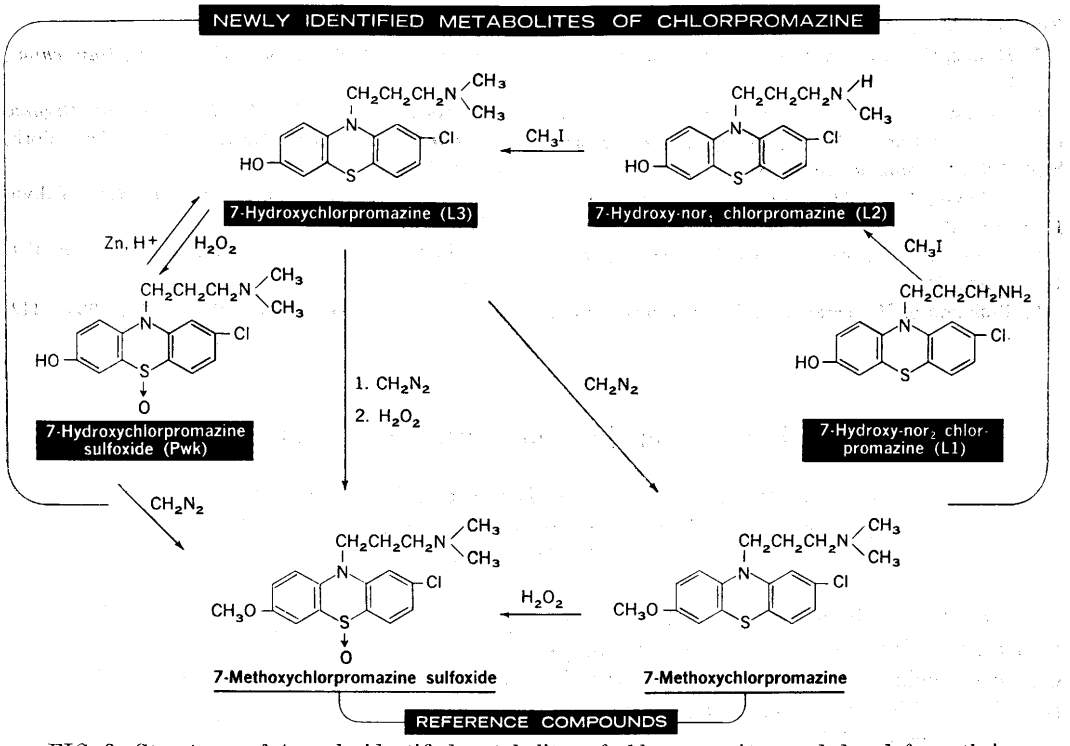


FIG. 2. Structures of 4 newly identified metabolites of chlorpromazine, as deduced from their chemical reactions, spot tests and paper chromatographic behavior. See Tables I and II and text.

the urine of dogs, and still more in human urine. We have already pointed out that hydroxylation and glucuronide formation are species-dependent, and represent a more important pathway of biotransformation in man than in the dog(4). Work is in progress to identify those human hydroxychlorpromazines which are not seen in dog urine.

Investigations at the Hillside Hospital and elsewhere attest to the wide variation in response to chlorpromazine. This variation is first apparent in minimum dosage required for symptomatic relief from agitation, and later in the individual pattern of response to the drug. The present studies were initiated to determine whether the clinical reaction to chlorpromazine bears a relation to the pattern of its metabolism and to subsequent disposition of its metabolites. Further studies may also throw light on the genetic and prognostic significance of variations in chlorpromazine metabolism in mental disorders.

*Summary.* A family of 4 phenolic metabolites of chlorpromazine has been isolated from animal (dog) and human urine by solvent extraction and paper chromatography. By subjecting the metabolites to a series of methylation and oxidation or reduction reactions, then comparing the products obtained to authentic material, they have been identified as follows: 7-hydroxychlorpromazine, 7-hydroxychlorpromazine sulfoxide, 7-hydroxy-nor$_1$chlorpromazine, and 7-hydroxy-nor$_2$chlorpromazine. These compounds are excreted both in free form and as glucuronic acid conjugates.

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1. Nadeau, G., Sobolewski, G., *Canad. Med. Assn. J.*, 1959, v80, 826.

2. Lin, T. H., Reynolds, L. W., Rondish, I. M., Van Loon, E. J., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 602.

3. Posner, H. S., *Abst. A.C.S. Meeting*, Sept. 1959, p81C, paper 179.

4. Goldenberg, H., Fishman, V., PROC. SOC. EXP.

BIOL. AND MED. 1961, v108, 178.

5. Huang, C. L., Kurland, A. A., *Clin. Chem.*, 1961, v7, 574.

6. Forrest, I. S., Piette, L. H., 3rd Congress Collegium Internationale Neuropsychopharmacologicum, Sept. 1962, Communications, p52.

7. Fishman, V., Goldenberg, H., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 99.

8. Fishman, V., Heaton, A., Goldenberg, H., *ibid.*, 1962, v109, 548.

9. Tsukamoto, H., Terada, S., *Chem. Pharm. Bull.*, Tokyo, 1962, v10, 91.

10. Boer, T. J. de, Backer, H. J., *Rec. trav. chim.*, 1954, v73, 229.

11. Hickinbottom, W. J., *Reactions of Organic Compounds*, Longmans, Green & Co., New York, 1957, p408.

12. Kano, H., Fujimoto, M., *Pharm. Bull.*, Tokyo, 1957, v5, 389.

13. Platonow, N., Oliver, W. T., *Research Vet. Sci.*, 1960, v1, 371.

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## Dose-Response Relationship in Aminonucleoside Nephrosis.* (28089)

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A possible mode of action of the aminonucleoside† (PA) of the antibiotic puromycin in inducing the nephrotic syndrome in rats is by blocking nucleic acid synthesis through enzyme inhibition(1-3). To test this hypothesis we have administered decreasing doses of PA subcutaneously at weekly or biweekly intervals instead of the usual daily dose of 1.5 mg/100 g. It was reasoned that the appearance of proteinuria under these circumstances would constitute evidence that *direct* enzyme inhibition was not involved, since at these low doses sufficient blood levels to block a postulated enzyme system would likely not be maintained, especially over such a protracted period. By varying the dose schedules in this manner, by administering PA into the circulating blood and by utilizing "stopped" schedules (1-4 consecutive daily doses), we found, unexpectedly, that the development of proteinuria depended not only on the total cumulative dose administered but also on reaching a certain threshold dose.

*Methods.* Female albino rats of the Holtzman strain weighing 200 ± 20 g were divided into groups of 3-6 rats each. Doses of PA ranging from 1-3 mg were administered subcutaneously, intravenously, or into the heart or aorta according to the schedule shown in Table I. For intravenous and intracardiac injections the rats were lightly anesthetized with ether. For intra-aortic administration, the drug was injected just above the renal arteries through a 26-gauge needle while the aorta at its bifurcation was temporarily obstructed by finger compression. The drug was prepared as a 0.50% solution in distilled water or isotonic saline and the actual volume injected was always less than 1 ml. Urine was collected daily or weekly and 24-hour protein concentrations were determined by the method of Shevsky and Stafford as modified by McKay(4).

TABLE I. PA Dosage Schedule.

| Group | No. rats | Dose/rat (mg) | Route of admin | Interval |
|---|---|---|---|---|
| A | 3 | 3 | Subcutaneous | Weekly |
| B | 3 | 3 | " | Biweekly |
| C | 3 | 2 | " | " |
| D | 4 | 1 | " | " |
| E | 6 | 3 | " | Daily × 16 |
| F | 8 | 20 | Intravenous | Single dose |
| G | 4 | 3 | " | Daily × 6 |
| H | 4 | 5 | Intra-aortic | Single dose |
| I | 4 | 5 | Intracardiac | " " |
| J | 12 | 3 | Subcutaneous | Daily × 13 |
| K | 6 | 3 | " | Single dose, repeated at 4 weeks |
| L | 6 | 3 | " | Daily × 2 |
| M | 6 | 3 | " | " × 3 |
| N | 6 | 3 | " | " × 4 |

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† 9-(3'-amino-3'-deoxy-B-D-ribofuranosyl)-6-dimethyl aminopurine.