[CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1]]

Zymonic Acid, a New Metabolic Product of Some Yeasts Grown in Aerated Culture. I. Structure Studies[2]

BY FRANK H. STODOLA, ODETTE L. SHOTWELL AND LEWIS B. LOCKWOOD

RECEIVED JULY 2, 1952

A $C_8H_{10}O_5$ compound was obtained by the action of diazomethane on the acids from the yeasts *Trichosporon capitatum*, *Hansenula subpelliculosa* and *Kloeckera brevis*. Saponification data, conversion to an amide and dianilide, and a C-methyl determination showed this product to be the methyl ester of a furan α-carboxylic acid containing methyl and methoxyl groups as ring substituents. Ease of decarboxylation of the free acid indicated the methoxyl group to be beta to the carboxyl. On the basis of these studies the acid, as it exists in the culture liquor, is judged to be an equilibrium mixture of 3,5-diketo-4-methyltetrahydrofuroic acid and its enol.

The remarkable synthetic powers of the molds and bacteria have been amply demonstrated throughout the years by the isolation of numerous extracellular metabolic products. The abilities of the yeasts in this respect, however, have received scant consideration except for the well-studied alcoholic fermentation. Only a few scattered reports have appeared in the literature on the formation of ethyl acetate and such acids as formic, acetic, lactic, succinic and citric.

As a part of our search for new fermentation products, we have sought to fill, partially, this gap in our knowledge by surveying in aerated culture about 120 species of yeast from 24 different genera. About a dozen species produced acids in from 25–50% yields, and from this group we selected *Trichosporon capitatum* for detailed study.

This organism is a member of a highly oxidative genus showing some relationship to the molds. It undergoes the budding that is characteristic of the yeasts and, in addition, it has the capacity to produce oidiospores, which is typical of the mold genus *Geotrichum*. The strain used in our work was isolated in Sweden by Rennerfelt from a tree exudate and characterized as a new species by Diddens and Lodder in 1942.

The medium used in the fermentation contained 10% glucose, magnesium sulfate, potassium dihydrogen phosphate, corn steep liquor, urea and calcium carbonate. The fermentation flasks were kept on a rotary shaker until the glucose concentration fell to about 2%. This required about 11 days, and in that time about half of the glucose was converted into a mixture of calcium salts. The calcium ion was removed with an ion-exchange resin and the resulting acid solution extracted with ether for 20 hours which removed about 50% of the acid present.

Preliminary studies on the ether-soluble acid fraction showed that it contained an unstable acid which resinified and evolved gas on standing at room temperature. To obtain a stable product the ether extract was treated with an excess of diazomethane. Distillation *in vacuo* gave as the main fraction a liquid methyl ester, the weight yield of which was about 14% based on the glucose consumed. Refractionation gave a pure product boiling at 120° at 1 mm. Structure studies on this methyl ester ($C_8H_{10}O_5$) have led us to assign formula I to it. Although we have not yet isolated the free acid in the pure form, we have been able to show that it probably exists in the culture liquor in the equilibrium forms represented by formulas II and III. The name "zymonic acid" has been assigned to this new fermentation acid to suggest its derivation from the yeasts.

Studies on the ester (I) showed the presence of two saponifiable groups and two methoxyl groups. Treatment of the ester with ammonia gave a crystalline monoamide (IV) ($C_7H_9O_4N$) which still contained one methoxyl group and one saponifiable group. No evidence for the presence of hydroxyl or carbonyl groups in the amide could be obtained.

Prolonged boiling of the ester (I) with strong alkali led to the formation of a crystalline product which proved to be the monohydrate of the monobasic acid $C_7H_8O_5$ (V). Like the amide, this acid retained one methoxyl group and one saponifiable group. Such behavior was judged to be compatible only with a structure having a lactone ring and a methoxy ether linkage.

It was demonstrated that the action of strong alkali on the ester (I) had brought about no change except saponification of the ester grouping. This was done by conversion of the acid (V) to the amide (IV) *via* the acid chloride.

A Kuhn–Roth determination on the amide (IV) showed the presence of a C-methyl group, thereby limiting the size of the ring to that of a γ-lactone. Apparently the ring is very stable since no evidence was found of ring opening on titration of the acid (V). When the lactone ring was opened with strong sodium hydroxide solution and the disodium salt then acidified, there was complete reversion to the lactone form.

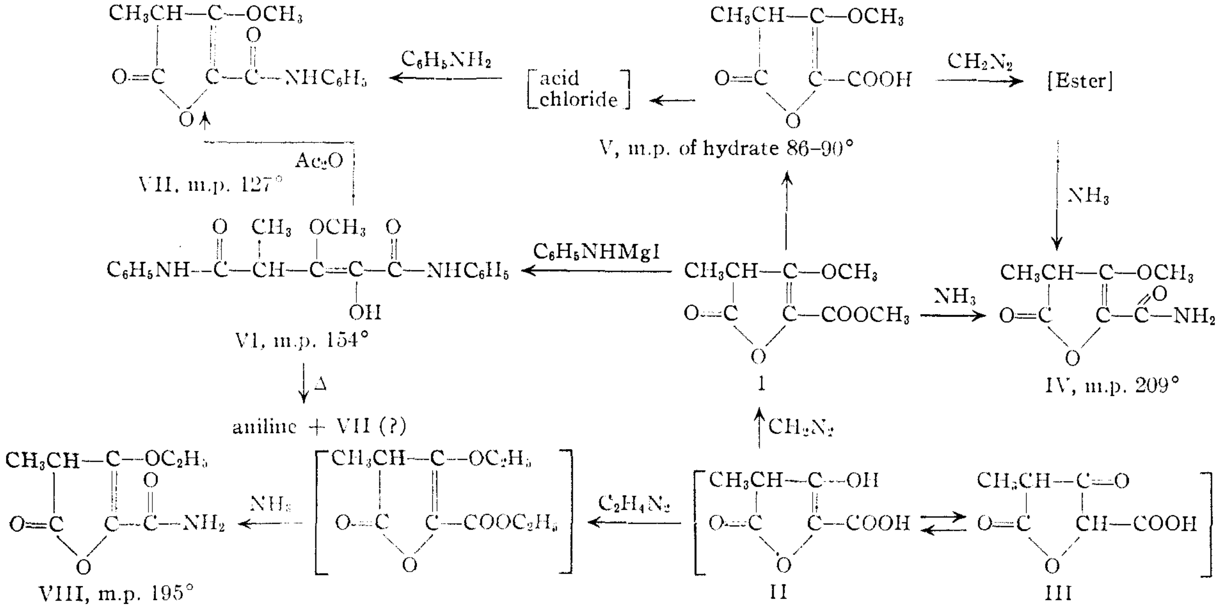
In accordance with the lactone structure, the methyl ester (I) could be converted in good yield by reaction with anilinomagnesium iodide into a crystalline compound which showed analytical figures in agreement with the dianilide formula VI. Even this dianilide showed a strong tendency toward ring closure. Treatment with acetic anhydride in pyridine at room temperature was sufficient to convert it to the same monoanilide (VII) as that obtained from the acid (V). Likewise when the dianilide (VI) was heated it liberated aniline, presumably with the formation of the monoanilide (VII).

The relative positions of the methoxyl and

---

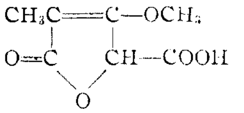
[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

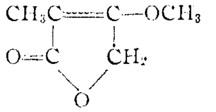
[2] Presented before the Division of Biological Chemisty at the 12th International Congress of Pure and Applied Chemistry, New York, N. Y., September, 1951.

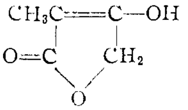


carboxyl groups in the acid (V) was determined by means of the following reactions: Acid hydrolysis of the acid (V) yielded 1 mole of carbon dioxide which indicated the presence of a methyl ether of the enol form of a β-keto acid. Treatment of the acid (V) with concentrated sulfuric acid at 110° gave 1 mole of carbon monoxide, whereas the amide (IV) gave none. Since such elimination of carbon monoxide is characteristic of α-hydroxy or α-keto acids, opening of the lactone ring must have taken place during the heating with sulfuric acid. Tetrahydrofuroic acid was shown to yield 1 mole of carbon monoxide under the same conditions.

These considerations left only the formula V or that of a tetronic acid (IX) as possible structures for the acid.


IX


X


XI

Formula IX was considered to be untenable when it was found that the model compound (X) was readily hydrolyzed to the enol XI by water at room temperature, a behavior not shown by the acid (V). Furthermore compound X showed a single band absorption at 2670 Å. compared to the value of 2340 Å. for the acid (V). The β-methoxy acrylic acid structure assigned to acid (V) is in accord with the finding of Bowden, *et al.*,[3] that the compound $CH_3O(CH_3)C{=}CHCOOH$ shows a single band at 2340 Å.

Attempts to isolate the free zymonic acid as it exists in the acidified culture liquor were not successful. It remained, therefore, to establish whether the ether methoxyl was present as such or was introduced by reaction of the diazomethane with an enol. This point was settled as follows: A sample of the ether-soluble acids, alkylated with diazoethane, yielded the ethoxy amide (VIII) after ammonolysis of the purified ester. Such a product could have resulted only if zymonic acid contains a free enolic group.

It was of interest to determine whether other species of yeasts would produce zymonic acid when grown in aerated culture. Accordingly, we next investigated a member of the genus *Hansenula*, a group of yeasts totally unrelated morphologically to *Trichosporon capitatum*. For this work we used a non-sporogenous form of *Hansenula subpelliculosa*, a species first described by Bedford.[4]

Grown under the same conditions as already described for *Trichosporon capitatum*, this strain of *Hansenula* produced a good yield of ether-soluble acids from which the methyl ester was prepared with diazomethane. Fractionation and treatment with ammonia gave the amide IV. Similarly a strain of *Kloeckera brevis* also gave the amide IV in good yield.

The fact that zymonic acid is produced by such widely different yeasts suggests that it might be part of a common pathway in the breakdown of glucose by this class of microörganisms. The branched chain of zymonic acid, of course, indicates that the glucose is first dismutated to three-carbon fragments such as pyruvic and glyceric acid which are then recombined to form zymonic acid. Since zymonic acid is a β-keto acid, it may be that it occupies a place analogous to oxalsuccinic acid of the Krebs cycle. So far the instability of oxalsuccinic acid has prevented its isolation from living systems. It appears that the greater stability of zymonic acid resulting from the presence of a ring permits its accumulation in the culture liquor.

Experimental

All melting points are capillary and corrected.

Methyl Ether of Methyl Zymonate from ***Trichosporon capitatum*****.**—The yeast (NRRL Y-1487) was grown on a

---

(3) K. Bowden, E. A. Braude and E. R. H. Jones, *J. Chem. Soc.*, 949 (1946).

(4) C. L. Bedford, *Mycologia*, 34, 628 (1942).

"Medium D" having the following composition per liter: 97.1 g. of glucose, 0.6 g. of KH_2PO_4, 0.25 g. of $MgSO_4 \cdot 7H_2O$, 2.5 g. of corn steep solids, 40 g. of $CaCO_3$ and 2.0 g. of urea. The last two ingredients were sterilized separately. Each erlenmeyer flask (300 ml.) contained 125 ml. of culture liquor and was shaken on a rotary shaker at 28–29°. At the time of harvest (11 days) the glucose concentration had fallen to 18.7 g. per liter. The Seitz-filtered culture liquor showed 8.84 g. of calcium per liter. Concentration of an aliquot to dryness gave a value of 77.0 g. of solids per liter which corresponded to 58.3 g. of calcium salt after correction for residual glucose.

The culture liquor (1810 ml.) was freed of cations with an exchange resin and the eluate concentrated *in vacuo* to 300 ml. Continuous ether extraction for 16 hours gave an ether solution which contained 46.3 g. of acid which was about 52% of the total acid calculated to be present.

The ether extract, which gave a red color with ferric chloride, was treated with excess diazomethane and the methyl esters fractionated in an efficient still. The main fractions were (A) 9.51 g., $n^{27}\text{D}$ 1.4589; (B) 10.87 g., $n^{27}\text{D}$ 1.4658, C, 51.4; H, 5.6. Refractionation of fraction A gave 4.25 g. of pure material, b.p. 118–123° (1 mm.), $n^{28}\text{D}$ 1.4640.

Rotation: 81.5 mg. made up to 1.384 ml. with dimethylformamide at 27° gave αD +0.07°, *l* 1; $[\alpha]^{27}\text{D}$ +1.2°.

Anal. Calcd. for $C_8H_{10}O_5$: C, 51.6; H, 5.41; OCH_3, 33.3; mol. wt., 186; sapn. equiv., 93. Found: C, 51.6; H, 5.71; OCH_3, 31.2; mol. wt. (Rast), 189; sapn. equiv., 99.

Low methoxyl values on the esters were encountered throughout this work and appear to be characteristic of these compounds. Hydrogenations in 95% alcohol with large amounts of platinum catalyst gave hydrogenation numbers varying from 341 to 468. In 95% alcohol the ester showed a single band at 2310 Å. ($E^{1\%}_{1\text{cm}}$ 319, ϵ 5940).

A fermentation conducted about a year later than the one described above, gave a methyl ester fraction from which the pure methyl ether of zymonamide could be obtained in good yield. We had no difficulty in reproducing the results of this fermentation after it was learned that the glucose concentration should not be allowed to fall below about 2%. Fermentations carried to the point of 0.1% glucose apparently contained only trace amounts of zymonic acid.

Methyl Ether of Zymonamide (IV).—The *Trichosporon* ester (504 mg. of the 4.25-g. analytical sample) dissolved in 2 ml. of absolute alcohol and 5 ml. of liquid ammonia was kept in a bomb at room temperature for four days. The 344 mg. of crystals obtained were crystallized from acetone–petroleum ether, yield 277 mg., m.p. 209–210°.

Anal. Calcd. for $C_7H_9NO_4$: C, 49.1; H, 5.30; N, 8.19; OCH_3, 17.9; mol. wt., 171. Found: C, 49.2; H, 5.26; N, 8.16; OCH_3, 17.6; mol. wt. (Signer), 165.

The amide crystallized unchanged after solution in boiling acetic anhydride. It does not form a carbonyl derivative with 2,4-dinitrophenylhydrazine. A Kuhn–Roth determination gave 0.84 mole of acetic acid per mole of amide. In the ultraviolet the compound showed a single peak at 2330 Å. in 95% alcohol ($E^{1\%}_{1\text{cm}}$ 448, ϵ 7670).

Ethyl Ether of Zymonamide from *T. capitatum*.—A fermentation in which 87 g. of glucose was consumed gave 19.3 g. of ether-soluble acids on 16 hours of extraction. Treatment with excess diazoethane and fractionation gave 2.45 g. of ethyl ester. This ester (1.34 g.) yielded 230 mg. of crude crystalline amide on reaction with ammonia in absolute ethanol at room temperature. Crystallization from acetone–petroleum ether gave 202 mg., m.p. 195.5–196.5°.

Anal. Calcd. for $C_8H_{11}O_4N$: C, 51.9; H, 5.99; N, 7.57; OC_2H_5, 24.3. Found: C, 52.3; H, 5.99; N, 7.43; OC_2H_5, 24.1.

The melting point on admixture with the methyl ether of zymonamide was 180–185°.

Methyl Ether of Zymonic Acid.—The methyl ester (1.01 g.) was shaken with 10 ml. of water containing 1 g. of KOH until a clear solution resulted. After standing at room temperature for two days, the solution was acidified and extracted with ether. Evaporation of the ether left 944 mg. (91% of theory) of the crystalline hydrate, m.p. 50–70°. This product (150 mg.) was dissolved at 90° in 6 ml. of *n*-butyl ether saturated with water. The clear solution, pipetted from a small amount of gummy material, deposited arborescent crystals on standing at room temperature; 79 mg., m.p. 86–90° (vigorous evolution of gas at 110–120°). The lead, silver, copper, barium, zinc and mercuric salts were all soluble in water.

In alcohol the acid showed a single peak at 2340 Å. ($E^{1\%}_{1\text{cm}}$ 289, ϵ 5500).

The air-dried product lost only 8.7% of its weight on drying to constant weight at 1 mm. at room temperature (calculated for a monohydrate 9.47%). The following analyses are based on a sample dried in that manner.

Anal. Calcd. for $C_7H_8O_5$: C, 48.8; H, 4.69; OCH_3, 18.0. Found: C, 49.2; H, 4.32; OCH_3, 18.3.

Vigorous saponification with 20% potassium hydroxide solution at 100° for 17 hours gave the same acid hydrate in good yield. To ensure that this treatment had induced no change other than the saponification of the ester grouping, the acid hydrate was converted to the methyl ester with diazomethane and then to the amide. Comparison by a mixed melting point showed this amide to be identical with the amide obtained directly from the ester I with liquid ammonia in alcohol.

A quantitative α-hydroxy acid determination on the acid hydrate with concentrated sulfuric acid at 110° showed the evolution of 1.118 ml. (S.T.P.) of carbon monoxide from 10.37 mg. of the acid hydrate which corresponds to 0.92 mole per mole of hydrate. Under the same conditions the amide IV gave no carbon monoxide.

Thermal decomposition (125°) of the acid hydrate (190 mg.) yielded 36.13 mg. of carbon dioxide or 82% of that calculated for the decomposition of a monocarboxylic acid.

Refluxing of the acid hydrate (40.55 mg.) with 3 *N* sulfuric acid gave the following weights of carbon dioxide (the yields are the per cent. for elimination of carbon dioxide from one carboxyl group):

Total reflux time, hr.	Total weight of carbon dioxide, mg.	Yield, %
4	5.16	55
12	9.03	95
23	10.43	110
33	10.92	115
56	11.64	123

Methyl Ether of Zymonanilide (VII).—The acid hydrate (266 mg.) was refluxed for five minutes with 2 ml. of thionyl chloride. Removal of the excess reagent and treatment of the residue with excess aniline gave 244 mg. of crude anilide (70% of theory). Crystallization from 80% alcohol and from ethanol-petroleum ether gave 63 mg. of feathery crystals, m.p. 127–127.5°. For analysis the compound was dried at 56° *in vacuo*.

Anal. Calcd. for $C_{13}H_{13}NO_4$: C, 63.2; H, 5.30; N, 5.67; OCH_3, 12.6. Found: C, 63.6; H, 5.39; N, 5.57; OCH_3, 12.9.

In 95% alcohol the anilide showed a single peak at 2390 Å. ($E^{1\%}_{1\text{cm}}$ 667, ϵ 16,490).

Dianilide (VI) from the Methyl Ester (I).—The anilinomagnesium iodide reagent was prepared in 50 ml. of dry ether from 1 g. of magnesium, 2.37 ml. of ethyl iodide and 4 ml. of aniline. To this solution was added 400 mg. of the methyl ester and the reaction mixture refluxed for ten minutes. After acidification with hydrochloric acid, the dianilide was removed by ether extraction, yield 318 mg. (44%). Crystallization from dilute acetone gave blocks, m.p. 154.3–155.8°.

Anal. Calcd. for $C_{19}H_{20}O_4N_2$: C, 67.1; H, 5.92; N, 8.23; OCH_3, 9.1. Found: C, 67.0; H, 5.98; N, 8.00; OCH_3, 9.1.

In 95% alcohol the dianilide gave a broad band at 2500 Å. ($E^{1\%}_{1\text{cm}}$ 654, ϵ 22,240).

When heated at 185° (11 mm.) 25 mg. of the dianilide gave a distillate of aniline which was converted to 19 mg. (79% yield) of crude 2,4,6-tribromoaniline. Sublimation yielded 14.5 mg. of pure product (calcd.: N, 4.50; found: N, 4.24;) m.p. 120.2–120.7°. This melting point was not depressed on admixture with an authentic sample.

An attempt to acetylate the dianilide led to the formation of the methyl ether of zymonanilide (VII). Carefully purified dianilide (158 mg.) was dissolved in 1 ml. of pyridine and 1.5 ml. of acetic anhydride and allowed to stand overnight at room temperature. Addition of water, neutralization, and extraction with ether gave 181 mg. of a gum which slowly crystallized. Repeated crystallization from various solvents gave 45 mg. of needles, m.p. 125.4–126.4°, which were identified by elementary analyses, mixed melting point, and X-ray diffraction pattern as the methyl ether of zymonanilide (VII).

Anal. Calcd. for $C_{13}H_{13}NO_4$: C, 63.2; H, 5.30; N, 5.67; OCH_3, 12.6. Found: C, 63.4; H, 5.28; N, 5.50; OCH_3, 12.5.

Permanganate Oxidation of Methyl Ether of Zymonic Acid.—The acid hydrate (653 mg.) was dissolved in 36 ml. of ice-cold sodium bicarbonate solution (2.5%). Over a period of 40 minutes a potassium permanganate solution (733 mg. in 28 ml. of water) was added dropwise with shaking. After dilution with water, the solution was heated to coagulate the manganese dioxide which was then removed by filtration. The *p*H of the filtrate was adjusted to 6 and a calcium chloride solution (1 g. in 5 ml. of water) added. The calcium oxalate (289 mg.) was separated, treated with hydrochloric acid, and the oxalic acid removed by ether extraction (227 mg. anhydrous acid or 73% of theory). The oxalic acid was converted to the dibenzylamine salt, m.p. 205–206°, which showed no depression in melting point on admixture with an authentic sample.

Methyl Ether of Tetrinic Acid (X).—Tetrinic acid (XI), prepared by the method of Wolff,[5] was converted with diazomethane into the methyl ether which has been reported as a liquid by Conrad and Gast.[6] By fractionation we obtained one of the isomers, presumably the *trans* form, as a crystalline solid, m.p. 84–85° (long needles from acetone-petroleum ether).

Anal. Calcd. for $C_6H_8O_3$: C, 56.2; H, 6.29; OCH_3, 24.2. Found: C, 55.9; H, 6.00; OCH_3, 24.5.

In 95% alcohol the compound showed a single band at 2670 Å. ($E^{1\%}_{1\text{cm}}$ 1436, ε 18,380). It was completely hydrolyzed by water on the steam-bath in five minutes or at room temperature overnight. Correct methoxyl values were obtained only when the hydriodic acid was cooled in ice before the sample was added.

Methyl Ether of Zymonamide from *Hansenula subpelliculosa*.—A fermentation using this organism (NRRL Y-1009) was conducted in the same manner as with *Trichosporon capitatum*, except that "Medium B" was used (same as Medium D only the urea was replaced by 2.5 g. $(NH_4)_2SO_4$). This yielded a methyl ester fraction (b.p. 117–118° at 1 mm., $n^{28}D$ 1.4651) which gave a crystalline amide in good yield. This product (m.p. 208–209°) analyzed as follows.

Anal. Calcd. for $C_7H_9O_4N$: C, 49.1; H, 5.30; N, 8.19; OCH_3, 17.9. Found: C, 49.4; H, 5.18; N, 8.30; OCH_3, 18.5.

This sample was shown by mixed melting point and by comparison of X-ray patterns to be identical with the amide from *Trichosporon capitatum*.

Methyl Ether of Zymonamide from *Kloeckera brevis*.—From a fermentation using this organism (NRRL Y-915) on Medium B, 5.74 g. of methyl ester (b.p. 112–117° at 1 mm., $n^{28}D$ 1.4650) was obtained from 56 g. of glucose (10.3% weight yield). The ester gave in good yield an amide shown by mixed melting point to be identical with the *Trichosporon* derivative.

PEORIA, ILLINOIS

---

[5] L. Wolff, *Ann.*, **288**, 16 (1895).

[6] M. Conrad and R. Gast, *Ber.*, **31**, 2731 (1898).

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF TEMPLE UNIVERSITY]

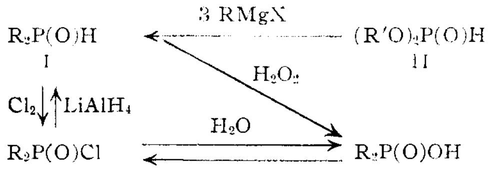
Di-*n*-alkylphosphine Oxides. I. Synthesis

BY ROBERT H. WILLIAMS[1a] AND LYLE A. HAMILTON[1b]

RECEIVED APRIL 18, 1952

The preparation of the di-*n*-hexyl-, di-*n*-octyl- and di-*n*-octadecylphosphine oxides, *i.e.*, compounds of the type $R_2P(O)H$, by the reaction of the appropriate Grignard reagent with di-*n*-butyl phosphite is reported. The di-*n*-octylphosphine oxide has also been prepared by the reaction of di-*n*-octylphosphinyl chloride, $(n\text{-}C_8H_{17})_2P(O)Cl$ with lithium aluminum hydride. These higher di-*n*-alkylphosphine oxides are white solids and are stable, neutral entities. Chemical evidence suggests that they exist as the phosphine oxides rather than the isomeric phosphinous acids, R_2POH.

A review of the chemical literature has revealed no description of the di-*n*-alkylphosphine oxides (phosphinous acids). We have been able to prepare the di-*n*-hexyl-, di-*n*-octyl- and di-*n*-octadecylphosphine oxides. This preparation is most conveniently accomplished by treating the appropriate Grignard reagent with di-*n*-butyl phosphite or other dialkyl phosphite, II, although the replacement of the halogen in a dialkylphosphinyl chloride (R_2P-$P(O)Cl$), III, with hydrogen, using lithium aluminum hydride, has also been successful. We have been unable to isolate either dimethylphosphine oxide or diphenylphosphine oxide from the reaction of the appropriate Grignard reagent on di-*n*-butyl phosphite, probably because of the greater instability of these phosphine oxides.



As proof of structure of the dialkylphosphine oxides, the di-*n*-octylphosphine oxide was chlorinated to the phosphinyl chloride and the latter hydrolyzed to the phosphinic acid. This acid was identical with di-*n*-octylphosphinic acid prepared by the addition of *n*-1-octene to hypophosphorous acid.[2] Direct oxidation of the di-*n*-octylphosphine oxide with hydrogen peroxide gave the same di-*n*-octylphosphinic acid. The conversion of dialkyl phosphites, II, to dialkylphosphinic acids, IV, by reaction with Grignard reagent and oxidation with hydrogen peroxide but without isolation of intermediates, has been reported by Kosolapoff.[3] The method of synthesis of di-*n*-octylphosphine oxide from the phosphinic acid by conversion of the latter to the phosphinyl chloride and replacement of the chlorine with hydrogen by means of lithium

---

[1] (a) Socony-Vacuum Fellow at Temple University; (b) present address: Socony-Vacuum Laboratories, Paulsboro, New Jersey.

[2] British Patent 660,918.

[3] G. M. Kosolapoff and R. M. Watson, THIS JOURNAL, **73**, 4101 (1951).