636. *Humulinone: its Alleged Occurrence in Hops.*

By M. Verzele and F. Govaert.

Recently Cook and Harris (*J.*, 1950, 1873) obtained a strongly acidic compound (pK 2·7), which they called "humulinone," by treatment of the light-petroleum soluble hop resins with either saturated aqueous sodium hydrogen carbonate (giving the sodium salt) or 90% methanolic lead acetate (a mixture of the lead salts of humulone and humulinone then being obtained). These authors claimed that humulinone was present as a constituent of the hop resins, but its existence in hops, in the quantities reported, is incompatible with the results of hop analyses previously reported by us (*Congr. Intern. Inds. Fermentation*, 1947, 279; *Fermentatio*, 1948, **1**, 1).

In the first of our methods the chromatographically purified extract is treated with lead acetate, thus precipitating the α-acid, and the β-acid content is determined by potentiometric titration of the mother-liquor. In the second method the α-acid content is determined polarimetrically and the β-acid content from the total acidity, by difference. The results for α-acid content, obtained by use of the two methods, have always agreed, but this could not be so if humulinone were present as a constituent, for in the first method it would be precipitated with humulone and therefore not measured in the lupulone fraction, whereas in the second method the lupulone content would be augmented by that of humulinone.

If humulinone occurs naturally as a constituent of the hop resins it should be obtained from a methanol–water (1 : 1) solution of hop extract by extraction at pH 3·5, to remove neutral products, humulone (pK 5·4), and lupulone (pK 6·0), followed by adjustment of the pH to $<2·0$, and re-extraction. No humulinone could, however, be obtained by this method, although our material gave humulinone readily when Cook and Harris's method was used.

Humulinone is strongly retained on silica gel (15 g. of gel retain about 700 mg. of the compound), from which it cannot be eluted completely with benzene but should be removed by ether. If present in hops, humulinone would therefore be retained on the column when chromatographic purification was used. Elution in this way and treatment of the eluate with ether and aqueous sodium hydrogen carbonate gave no humulinone, provided that all humulone had been eluted previously (lead acetate test).

The only remaining possibility is that humulinone is an artefact, produced by oxidation of humulone after extraction from the hops. This is proved to be the case. Pure humulone remains unchanged in pure ether and sodium hydrogen carbonate solution in the absence of oxygen, but if the ether contains peroxides humulinone is obtained in yields of up to 50%. The formation of humulinone can be brought about in pure ether by small additions of perhydrol. The oxidation is catalysed by light petroleum [this catalytic influence of light petroleum on the oxidation of bitter acids is well known (cf. Lundin, *Wallerstein Lab. Comm.*, 1947, **10**, 231)], and traces of this were probably still present in the oils (obtained by evaporation of light petroleum extracts of the hop resins) from which Cook and Harris obtained humulinone.

Examination of several old hop samples indicated that humulinone was not formed during storage; it could not be isolated provided that all oxidising agents were carefully avoided during the treatment of the resin extracts with ether and sodium hydrogen carbonate solution.

Humulinone is not precipitated from methanol by lead acetate (cf. Cook and Harris, *loc. cit.*); indeed normal precipitation of humulone is hampered by the presence of humulinone.

We find that humulinone has no optical activity and there is evidence that it may be a mixture of three isomers.

Experimental.—Materials. Pure humulone was obtained by chromatographic purification of hop extract, as described in previous papers from this laboratory; it had m. p. 65—66° (phenylenediamine complex, m. p. 115—117°). The ether used was purified by washing, drying,

and fractionating the commercial material. " Enriched " ether was obtained by four-fold concentration of commercial ether which had been stored for long periods in clear-glass bottles. Sodium hydrogen carbonate solutions were prepared with water which had been boiled.

Experiments with humulone. (*a*) Pure humulone (1 g.), pure ether (25 c.c.), and sufficient sodium hydrogen carbonate solution (about 30 c.c.) to fill the flask were kept for 7 days out of contact with air. No apparent change occurred and the optical rotation of the product represented 975 mg. of humulone (humulinone having no optical activity).

(*b*) When the pure ether was replaced by " enriched " ether, carbon dioxide was evolved, and the sodium salt (384 mg.) of humulinone was obtained; this gave humulinone (327 mg.), m. p. 70—74°. No humulone was detected.

(*c*) The use of " enriched " ether and perhydrol (2·5 c.c.) led to only a small yield of humulinone (26 mg.); no humulone could be detected. A smell of fatty acids suggested that the oxidation had proceeded too far.

Experiments with hop extracts. The optical rotation of the hop extract indicated 37·2 g./l. of α-acid.

(*a*) When oxygen was rigorously excluded from a mixture of the extract, pure ether, and sodium hydrogen carbonate solution, no humulinone was obtained and the optical rotation showed no appreciable loss of humulone during 7 days.

(*b*) Treatment of the hop extract (25 ml.) with " enriched " ether and sodium hydrogen carbonate solution gave the sodium salt (464 mg., 45%) of humulinone. The residue had no optical activity.

(*c*) Addition of perhydrol (2 c.c.) to the solution as used in (*b*) resulted in a smaller yield of the salt (325 mg.). The residue had a small optical rotation.

(*d*) The yield of humulinone was very variable, the reaction easily proceeding too far. The highest yield was obtained by the addition of a few drops of perhydrol each day to the mixture until humulinone ceased to separate. In this way 2·38 g. of α-acid with 50 c.c. of ether and 50 c.c. of saturated sodium hydrogen carbonate solution gave 1·206 g. (49%) of the sodium salt of humulinone.

(*e*) An extract of hops (15 g.), from which α-acid had been completely removed by lead acetate treatment, did not give any humulinone on treatment with ether and sodium hydrogen carbonate solution.

Precipitations with lead acetate. (*a*) Treatment of a solution of α-acid (175 mg.) in methanol (10 c.c.) and water (1·75 c.c.) with 4%-methanolic lead acetate (4·60 c.c.) gave the lead salt (270 mg.) of humulone.

(*b*) When humulinone (100 mg.) was added to α-acid the lead salt (271 mg.) of humulone was again obtained, but (*c*) when the quantity of humulinone was doubled the amount of lead salt isolated fell (to 230 mg.).

(*d*) If the water was replaced by the same quantity of 0·3N-sodium hydroxide (enough to neutralise the humulinone) the yield of lead salt obtained in (*c*) rose to the normal value (268 mg.).

(*e*) Humulinone was not precipitated by lead acetate from methanolic solution.

Humulinone. Crude humulinone was adsorbed on a small column of silica gel and the chromatogram was well washed with benzene. The filtrate was concentrated and the residue crystallised about ten times from light petroleum and then from dilute methanol. The product had m. p. 104—105°, pK 2·6 (in 50% methanol) (Found: M, 378. Calc. for $C_{21}H_{30}O_6$: M, 378). The light petroleum mother-liquor yielded a second isomer, m. p. 70—71°, on evaporation. This had pK 2·5 (in 50% methanol) (Found: M, 381). Elution of the column with ether, removal of the solvent, and crystallisation of the residue from light petroleum–benzene and from dilute methanol gave a third compound, m. p. 182°, pK 2·8 (in 50% methanol) (Found: M, 380).

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637. *The Preparation and Stability of Hydroxymaleic Acid (Oxaloacetic Acid).*

By JOHN C. ROBERTS.

THE importance of oxaloacetic acid as an intermediate compound in animal and plant metabolism and its frequent use in biochemical investigations has necessitated a re-examination of the laboratory methods available for its preparation and its stability in the solid state. (The stability of the acid in aqueous solution has been investigated by Krebs, *Biochem. J.*, 1942, **36**, 303.)

Oxaloacetic acid is generally prepared by one of the following methods: (i) Malic acid is oxidised with hydrogen peroxide (Fenton and Jones, *J.*, 1900, **77**, 77). Reproducible results are difficult to obtain, the technique is laborious (necessitating a very large number of ether-extractions), and the yields are not good (Fenton and Jones claim a yield of " about 22% " of the presumably unrecrystallised acid). (ii) Ethyl oxaloacetate is hydrolysed with concentrated hydrochloric acid (Krampitz and Werkmann, *Biochem. J.*, 1941, **35**, 596). These authors do not state the yield. The present author, using a temperature of −20° for cooling the acid hydrolysis liquid, has obtained very poor yields (*ca.* 6% of unrecrystallised acid). It is possible that results are very greatly dependent on the purity of the starting materials (cf. Simon, *Compt. rend.*, 1903, **137**, 856). (iii) The pyridine salt of hydroxymaleic anhydride is decomposed with sulphuric acid (Wohl and Oesterlin, *Ber.*, 1901, **34**, 1144). Use of 12% sulphuric acid yields hydroxymaleic acid; 30% sulphuric acid gives hydroxyfumaric acid (Wohl and Lips, *Ber.*, 1907, **40**, 2294). These observations led Wohl and Claussner (*ibid.*, p. 2308) to the development of a method for preparing the two isomeric acids in a state of high purity but yields are poor. No precise yields are stated by Wohl and Claussner. The present author has obtained 27% of recrystallised hydroxyfumaric acid and 8% of recrystallised hydroxymaleic acid—yields calculated on the pyridine salt.

The following modification of the method of Wohl *et al.* yields very pure hydroxymaleic acid in much improved yield more reliably and less tediously.

Experimental.—Hydroxymaleic acid. Powdered tartaric acid (20 g.) is dissolved, at room temperature, in acetic anhydride (44 c.c.) containing concentrated sulphuric acid (0·6 c.c.) in a 100-c.c. flask fitted to a reflux condenser by a ground-glass joint. The solution is refluxed gently for 10 minutes, then cooled to room temperature, and crystallisation of the diacetyltartaric anhydride is induced by " scratching." After 1 hour at room temperature the crystals are filtered off (Whatman, No. 54 paper), pressed, washed with benzene (3 × 10 c.c.), pressed and drained thoroughly and kept in a vacuum over paraffin wax for 1½ hours. The yield of material of m. p. 129° is 23·0—24·2 g. (80—84%).

Diacetyltartaric anhydride (20 g.) is added, at room temperature, to *pure dry* pyridine (40 c.c.), in a thin-walled flask, and the mixture shaken vigorously. A pale green colour quickly develops whereupon glacial acetic acid (12 c.c.) is added, the whole warmed, with continous shaking, in warm (40°) water for 20 seconds, and the flask then immersed in crushed ice. Ice-cold *dry* ether (45 c.c.) is added, and the whole quickly mixed and immediately filtered. The pyridine salt of hydroxymaleic anhydride is pressed thoroughly, washed with absolute alcohol (3 × 8 c.c.), and then with *dry* ether (2 × 8 c.c.). The salt, m. p. 108—109° (decomp.), is very light greenish-brown and is preferably used without delay in the ensuing operation. The yield is somewhat variable (10·4—13·3 g.; average 12·0 g., 67%). (All the operations described in this paragraph must be performed without any delays.)

The pyridine salt of hydroxymaleic anhydride (12 g.) is added to ice-cold concentrated hydrochloric acid (18 c.c.). A dark green solution is produced which, after 15 minutes, begins to deposit dirty-white crystals. The mixture is left at −2° overnight. The crude oxaloacetic acid is filtered off (Whatman, No. 54 filter paper), pressed, washed with ice-cold 7N-hydrochloric acid (3 × 6 c.c.), well pressed, and drained. Finally, it is washed with ice-cold chloroform (3 × 5 c.c.) and again well pressed and drained. The crystals are kept in a vacuum-desiccator over phosphoric oxide and potassium hydroxide pellets for 1½ hours. The yield of crude oxaloacetic acid is 6·55 g. (80%).

The crude acid (6·5 g.) is dissolved in warm (50°) acetone (65 c.c.) and filtered through a plug of acetone-washed cotton-wool in a pre-warmed funnel. To the acetone solution is added, with stirring, warm (50°) chloroform (250 c.c.) and the mixture set aside until crystallisation is complete (about 1 hour). The crystals are filtered off, washed with a mixture (2 × 8 c.c.) of acetone (1 vol.) and chloroform (4 vols.) and dried in a vacuum-desiccator over phosphoric oxide for 1 hour. The product (4·4 g., 67%), m. p. 155°, is free from chlorides and, after having been dried for 6 hours, shows an equivalent of 66·1 (Calc. for $C_4H_4O_5$, as a dibasic acid: equiv., 66·04); the m. p. gradually falls and the equivalent increases—see below.

The overall yield of recrystallised hydroxymaleic acid, calculated on the tartaric acid used, is about 30%.

Stability. A sample of hydroxymaleic acid was prepared by the method of Wohl and Claussner (*loc. cit.*), recrystallised from acetone–benzene and stored in a vacuum-desiccator over phosphoric oxide for the first day, and, thereafterwards, in an ordinary desiccator over anhydrous calcium chloride. Alterations in m. p. and equivalent, occurring on storage, are recorded in the Table.

" Age " (hours)	0	0·5	1·75	3·5	23	56	80	120
M. p.[a]	157°	157°	153°	153°	152·5°	146·5°	146·5°	144°
Equiv.[b]	—	—	—	66·1	—	—	—	66·3

[a] M. p.s were determined with the bath heated at about 12°/minute. [b] Equivs. were determined by dissolution in ice-cold water and titration, immediately and rapidly, with 0·1N-sodium hydroxide (phenolphthalein). The first pink colour which persisted for 15 seconds was taken as the end-point.

If the fall of the m. p. and the increase of the equivalent weight are due to decarboxylation, the sample which had been stored for 5 days (equiv. 66·3) contained about 98·8% of hydroxymaleic acid (equiv., 66·04) and 1·2% of pyruvic acid (equiv., 88·06).

A sample of hydroxymaleic acid, m. p. 152—153°, " age " 3 hours, was decarboxylated with " aniline citrate " reagent (Edson, *Biochem. J.*, 1935, **29**, 2083), and the carbon dioxide produced (during 1 hour) was removed in a brisk current of carbon dioxide-free air, absorbed in " Sofnolite," and weighed. A purity of 98·5% was indicated. It appears that values obtained by the decarboxylation method are slightly low.

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638. *A Synthesis of (+)-β-Aminobutyric Acid from* L-*Alanine.*

By K. BALENOVIĆ, D. CERAR, and Z. FUKS.

β-AMINOBUTYRIC ACID was first resolved into its optical antipodes by Fischer and Scheibler (*Annalen*, 1911, **383**, 337) by using a very tedious procedure; the (+)- and the (−)-form showed $[\alpha]_D$ +35·3° and $[\alpha]_D$ −35·2°, respectively.

By application of the Arndt–Eistert reaction to (−)-1-diazo-3-phthalimidobutan-2-one, prepared from L-alanine (cf. *Experientia*, 1947, **3**, 369), (+)-β-aminobutyric acid, $[\alpha]_D$ +38·8°, has been obtained in five stages, in an overall yield of 38% (based on L-alanine).

Experimental.—N-*Phthaloyl-*L-*alanyl chloride.* *N*-Phthaloyl-L-alanine (3 g.; $[\alpha]_D^{18}$ −17·5°) (*Ber.*, 1907, **40**, 489) and thionyl chloride (6 c.c.) were heated for 1 hour at 60°; the excess of thionyl chloride was then removed under reduced pressure, the residue distilled, and the distillate (2·94 g., 92%), b. p. 186—192°/15 mm., 135°/1 mm., crystallised from light petroleum (b. p. 30—50°). The chloride had m. p. 38°, $[\alpha]_D^{17}$ −36·4°±0·5° (*c*, 0·20 in benzene) (Found: C, 55·9; H, 3·3. Calc. for $C_{11}H_8O_3NCl$: C, 55·6; H, 3·4%). Gabriel (*Ber.*, 1908, **41**, 247) reported m. p. 73° for the inactive form.

(−)-1-*Diazo-*3-*phthalimidobutan-*2-*one.* The chloride (6 g.) was dissolved in benzene (25 c.c.) and added to an ethereal solution of diazomethane (500 c.c.; obtained from 35 g. of nitrosomethylurea). After 24 hours the mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in the minimum quantity of dry ethyl acetate and precipitated with light petroleum. The (−)-1-*diazo-*3-*phthalimidobutan-*2-*one* (5·2 g., 85·2%), m. p. 88°, obtained was recrystallised from ethyl acetate–light petroleum; the m. p. remained unchanged, $[\alpha]_D^{18}$ −69·3°±1° (*c*, 0·48 in ethyl acetate) (Found: C, 59·5; H, 3·7. $C_{12}H_9O_3N_3$ requires C, 59·3; H, 3·7%).

Methyl (+)-β-*phthalimidobutyrate.* A freshly prepared alkali-free suspension of silver oxide (obtained from 0·5 g. of silver nitrate) was gradually added in four portions during 3 hours to a refluxing solution of the diazo-ketone (2·7 g.) in methanol (27 c.c.). The hot solution was treated with charcoal, filtered, and evaporated to dryness. The residue was extracted several times with light petroleum, the solvent removed from the combined extracts, and the product (1·8 g., 65%) repeatedly crystallised from light petroleum and finally sublimed at 110°/0·01 mm. *Methyl* (+)-β-*phthalimidobutyrate* had m. p. 38°, $[\alpha]_D^{16}$ +26·3°±1° (*c*, 0·26 in benzene) (Found: C, 62·9; H, 5·4. $C_{13}H_{13}O_4N$ requires C, 63·2; H, 5·3%).

(+)-β-*Aminobutyric acid.* A solution of the methyl ester (2 g.) in glacial acetic acid (5 c.c. and 50% hydriodic acid (6 c.c.) was heated under reflux for 7 hours. After cooling, the phthalic acid was filtered off and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in water, the solution extracted with ether (3 × 50 c.c.), and the water layer again evaporated to dryness. The crystalline hydriodide of (+)-β-aminobutyric acid was dissolved in water (400 c.c.) and passed through a column of Amberlite IR-4B (20—50 mesh; 15 g.) at a flow rate of 200 c.c./hour. The column was washed with distilled water (200 c.c.), and the washings were evaporated *in vacuo*. (+)-β-Aminobutyric acid (750 mg., 89%), m. p. 212°, was twice recrystallised from absolute methanol (the m. p. remained unchanged); it then had $[\alpha]_D^{18}$ +38·8°±1° (*c*, 0·48 in water), $[\alpha]_D^{19}$ +37·07°±1° (*c*, 6·0 in water) (Found: C, 46·3; H, 8·9. Calc. for $C_4H_9O_2N$: C, 46·6; H, 8·80%). Fischer and Scheibler (*loc. cit.*) reported $[\alpha]_D^{20}$ +35·3°±0·2° (*c*, 11·41 in water).

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639. *Mannich Bases of Acylaminomalonates, and a Synthesis of* DL-*Aspartic Acid.*

By R. O. ATKINSON.

IN connection with an investigation into possible methods of preparing certain β-substituted alanines, Mannich bases of ethyl acetamidomalonate and ethyl formamidomalonate were prepared. Butenandt and Hellmann (*Z. physiol. Chem.*, 1949, **284**, 168) prepared several of these compounds, using secondary amines of higher molecular weight [piperidine, morpholine, etc.], but they were unable to prepare the dimethylamino-compounds in crystalline form.

A procedure is described for preparing the dimethylaminomethyl compounds. The bases slowly decomposed when kept, but the readily prepared methiodides were quite stable.

Unlike Butenandt's (*loc. cit.*) piperidino-compound which, on acid hydrolysis, gave β-piperidylalanine, the dimethylamino-compounds gave glycine under the same conditions.

As expected, the esters would not react with sodium cyanide, the formation of an ethylenic intermediate being impossible. Snyder and Eliel (*J. Amer. Chem. Soc.*, 1948, **70**, 1073) and Snyder and Brewster (*ibid.*, 1949, **71**, 1058) have shown that in similar cases the necessary activation may be brought about by quaternisation.

Condensation of the methiodide with sodium cyanide proceeded vigorously in aqueous solution, and acid hydrolysis of the product gave aspartic acid in 71% yield.

Experimental.—Diethyl α-*acetamido*-α-*dimethylaminomethylmalonate.* 33% Dimethylamine solution [13·5 c.c., 0·1 mole] was treated at 0° with glacial acetic acid (15 c.c.). Diethylacetamidomalonate [21·7 g., 0·1 mole] and 40% formaldehyde solution [8·2 c.c.] were added and, after 30 minutes at room temperature, the mixture was cooled to −10° and made alkaline by slow addition of 20% sodium hydroxide solution. The Mannich *base* separated as white crystals (20 g., 73%), m. p. 50—51° (uncorr.) (Found: N, 10·1. $C_{12}H_{22}O_5N_2$ requires N, 10·2%).

The Mannich base (27·4 g., 0·1 mole) was dissolved in ethyl alcohol (60 c.c.), and methyl iodide (16 g., 0·11 mole) added; the *methiodide*, which crystallised spontaneously, was filtered

off after 30 minutes, washed with ether, and dried in air at 80° (19·6 g., 47·1%). It had m. p. 384° (uncorr.) (Found : N, 6·75; I^-, 31·1. $C_{13}H_{25}O_5N_2I$ requires N, 6·95; I^-, 30·5%).

Diethyl α-dimethylaminomethyl-α-formamidomalonate. Diethyl formamidomalonate (20·3 g., 0·1 mole) treated as described above gave the Mannich *base* as broken white plates (26 g., 100%), m. p. 77—78° (uncorr.) (Found : N, 10·6. $C_{11}H_{20}O_5N_2$ requires N, 10·7%). The base (26 g., 0·1 mole) with methyl iodide (16 g., 0·011 mole) gave the *methiodide* (19 g., 47·5%), m. p. 318—319° (uncorr.) (Found : N, 6·6; I^-, 31·2. $C_{12}H_{23}O_5N_2I$ requires N, 6·6; I^-, 31·7%).

DL-*Aspartic acid.* Diethyl α-dimethylaminomethyl-α-formamidomalonate methiodide (20·1 g., 0·05 mole) was added to a solution of sodium cyanide (4·9 g., 0·1 mole) in water (25 c.c.), and the mixture was warmed on a steam-bath; after a few seconds a vigorous reaction started and the solution became dark red. It was cooled for 5 minutes (the reaction becoming less vigorous), heated on a steam-bath for 3 hours, and then evaporated to dryness *in vacuo*. The residue was dissolved in hydrochloric acid (120 c.c.) and heated on a steam-bath for 6 hours. Evaporation to dryness *in vacuo*, and treatment of the alcoholic extract with pyridine gave the crude amino-acid, which was recrystallised from hot water. The yield was 4·7 g. (71%) (Found : C, 36·0; H, 5·2; N, 10·6. Calc. for $C_4H_7O_4N$: C, 36·1; H, 5·3; N, 10·5%). Partition chromatography on paper of the material and of an authentic sample of aspartic acid, followed by development with ninhydrin solution, gave identical R_F values for each sample.

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