*Chemical Constitution of Isorenieratene**

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The orange-colored sponge "*Reniera japonica*" contains a number of polyene pigments[1,2]. Among them a pigment named renieratene predominates in quantity, while a pigment of m. p. 199° forms a second constituent which amounts to 30~50 % of the former. It has been reported that this second constituent is also a hydrocarbon, $C_{40}H_{48}$ or $C_{40}H_{50}$, and exhibits the absorption maxima at 520, 484 and 452 mμ in carbon disulfide[2].

The absorption maxima and the melting point of the pigment closely resemble leprotin ($C_{40}H_{54}$, m. p. 199~200°; absorption maxima in carbon disulfide 517, 478, 447 mμ; 12 double bonds) which has been isolated by Grundmann and Takeda from an acid-resisting bacteria of a leper[3]. However, the pigment differs from leprotin in the molecular formula and in the number of double bonds. These differences seem to afford the evidence to conclude that these pigments are not identical with each other, though it may not be said that leprotin has been thoroughly characterized in these respects. Therefore the name *isorenieratene* is tentatively proposed to the pigment by the author.

The study of the chemical constitution of isorenieratene has been considerably difficult at first because of the small amount of material available (ca. 450 mg.), but as the pigment had fortunately a symmetrical structure which facilitated the isolation of the degradation products, it was possible to elucidate its constitution almost throughly, as will be described below.

The solution of the pigment isomerized by the addition of iodine shows the absorption spectrum with a marked *cis*-peak which can not be observed in the fresh solution (Fig. 1). Therefore isorenieratene seems to have an all-*trans* configuration as to the conjugated double bonds.

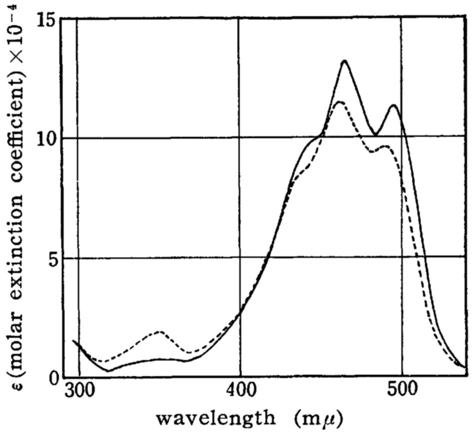

Fig. 1. Absorption curve of isorenieratene (benzene).
—— fresh solution
······ isomerized solution

Isorenieratene absorbs 15 moles of hydrogen on catalytic hydrogenation, which indicates that two rings exist in the molecule of the pigment. On determination of side chain methyl groups, it gives volatile acid corresponding to 7 moles of acetic acid.

The negative result in the determination of isopropylidene group indicates that isorenieratene possesses neither an isopropylidene group, nor an ionone ring. This agrees with the fact that the pigment has no Vitamin A activity.

From the molecular formula, isorenieratene is considered to be an isomer of renieratene which has a molecular formula $C_{40}H_{48}$ and 15 double bonds[4]. The results of the determinations described above are also very similar to those obtained on renieratene. Therefore isorenieratene is considered to have a structurally close relation to renieratene. In the previous paper[4] it has been interpreted that renieratene should have benzene rings in the molecule. The same argument is also valid for isorenieratene, and that the

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* "Pigments of Marine Animals", V. IV of this series; This Bulletin, **30**, 979 (1957).

[1] T. Tsumaki, M. Yamaguchi and T. Tsumaki, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zassi*), **75**, 297 (1954).

[2] M. Yamaguchi, This Bulletin, **30**, 111 (1957).

[3] C. Grundmann and Y. Takeda, *Naturwiss.*, **25**, 27 (1937); Y. Takeda and T. Ohta, *Z. Physiol. Chem.*, **258**, 6 (1939).

[4] M. Yamaguchi, This Bulletin, **30**, 979 (1957).

pigment possesses benzene rings as the terminal groups, is deducible from the following reasons. Though isorenieratene absorbs 15 moles of hydrogen, it exhibits the absorption maxima similar to those of β-carotene which has 11 double bonds. In order to contain fifteen unsaturations, a C_{40}-carotenoid should have nessesarily triple bonds, allenic bonds or ringsystems with benzenoid structure or the like. However, in the infra-red spectrum (Fig. 2) no bands are observed which indicate the presence of triple or allenic bonds, but on the other hand a marked absorption at 810 cm^{-1} is observed, which can be considered to be due to the out-of-plane deformation of two or three adjacent hydrogen atoms attached to the tetra- or tri-substituted benzene ring.

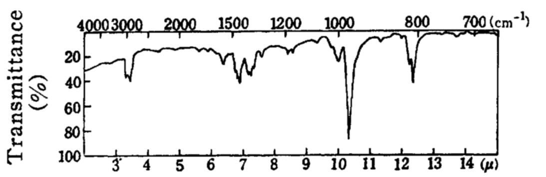

Fig. 2. Infra-red spectrum of isorenieratene.

The direct proof of the presence of benzene ring has been given by chromic acid oxidation and potassium permanganate oxidation. When isorenieratene is weakly oxidized with chromic acid corresponding to five atoms of oxygen per mole of the pigment, crocetin dialdehyde[5] (I) is obtained as a main product. This has established the structure of the middle part of the molecule of the pigment.

When isorenieratene is oxidized with chromic acid corresponding to two atoms of oxygen, a carotenoid aldehyde $C_{30}H_{36}O$ is obtained as a main product. The aldehyde is identical with isorenieral which has been obtained by the oxidation of renieratene[4]. From the fact that while renieratene gives two aldehydes, renieral and isorenieral, isorenieratene gives isorenieral only, it is concluded that isorenieratene has a symmetrical structure. From the colorless part of the chromatogram of the above oxidation product, a small amount of viscous oil is obtained. The oil affords the semicarbazone of m.p. 167°, which is proved to be identical with the semicarbazone of 2, 3, 6-trimethylbenzaldehyde (IV) described later.

When isorenieratene is oxidized with potassium permanganate, three acidic substances and one neutral product are obtained. One of the acidic products is oxalic acid (VII), which is identified by an elementary analysis and a mixed melting point. The second acidic product is a dibasic acid of m. p. 231°, which has a formula $C_{10}H_{10}O_4$. When the acid is further oxidized with potassium permanganate, it furnishes a tetrabasic acid of m. p. 215~238°, $C_{10}H_6O_8$, which is identified as 1, 2, 3, 4-benzenetetracarboxylic acid (VIII) by an elementary analysis and a mixed melting point with an authentic sample[6]. The dibasic acid of m. p. 231° is also formed synthetically by the moderate oxidation of 2, 3, 6-trimethylbenzoic acid (V), and it does not show any tendency to form an anhydride at the melting point. Therefore it is obvious that the acid is 2, 4-dimethylbenzene-1, 3-dicarboxylic acid (VI). As the third acidic substance is considerably difficult to purify because of the small amount of material and its large solubility, it is converted into *p*-phenylazophenacyl ester and purified by means of chromatography. The pure ester thus obtained is identical with the authentic sample of *p*-phenylazophenacyl-2, 3, 6-trimethylbenzoate (free acid: (V)).

The neutral fraction is a viscous oil and it gives impure semicarbazone, m. p. 149~154°. The semicarbazone is converted into 2, 4-dinitrophenylhydrazone, and the chromatographic purification of the latter gives a hydrazone of m. p. 219°. As it has been easily expected that the aldehyde may be 2, 3, 6-trimethylbenzaldehyde (IV), it has been synthesized from 3-bromopseudocumene by the Grignard reaction in a ca. 64% yield. The synthesized aldehyde gives 2, 4-dinitrophenylhydrazone of m. p. 219°, which is not depressed by mixing it with the sample from isorenieratene. The aldehyde gives a semicarbazone of m. p. 167°, which is proved to be identical with the sample obtained by the chromic acid oxidation of isorenieratene described above by the mixed melting point determination.

These results are summerized in Fig. 3, and it is concluded that isorenieratene has a structure of 1, 18-bis-(2, 3, 6-trimethylphenyl)-3, 7, 12, 16-tetramethyl-1, 3, 5, 7, 9, 11, 13, 15, 17-octadecanonaene (III), and that isorenieral has the structure indicated by the formula (II).

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[5] O. Isler, H. Gutmann, H. Lindlar, M. Montavon, R. Rüegg, G. Ryser and P. Zeller, *Helv. Chim. Acta*, **39**, 463 (1956).

[6] M. Freund and K. Fleischer, *Ann.*, **411**, 14 (1916).

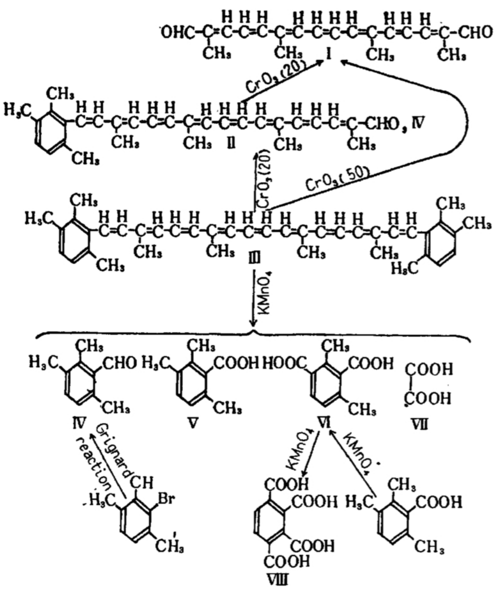
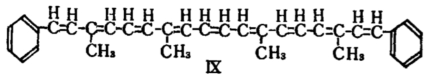
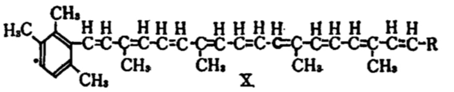


Fig. 3. Oxidation of isorenieratene.

A carotenoid with two non-substituted benzene rings (IX) has been synthesized by Eugster and coworkers[7], and it has been proved to exhibit the absorption maxima at 541, 502, 472 $m\mu$ in carbon disulfide. Isorenieratene however shows the absorption maxima at 520, 484, 452 $m\mu$ in the same solvent. The difference in the position of the absorption maxima is considered to be due to the steric hindrance caused by the methyl groups attached to the neighboring positions of the polyene chain in the benzene nucleus of the molecule of the pigment. It is considered that the presence of such methyl groups hinders the uniplanarity between the benzene rings and the polyene chain, and causes subsequent decrease in the degree of conjugation, which resulted in the shift of the absorption maxima to the shorter wavelength location[8].


IX

That the chemical constitution of isorenieral is expressed by the formula (II), lead to the conclusion that renieratene has a partial formula indicated by (X), for it has already been shown that renieratene gives isorenieral besides renieral on oxidation and that it is also the derivative of tetramethyloctadecanonaene.


X

The carotenoids have hitherto been considered to belong exclusively to aliphatic or alicyclic compounds. Therefore it should be emphasized that the presence of a carotenoid containing aromatic structure in nature is established for the first time by the elucidation of the chemical constitution of isorenieratene.

Isorenieratene can be regarded as composed of isoprene unites as indicated in Fig. 4, but it is also possible that the pigment is formed from β-carotene by the migration of methyl groups of 1- and $1'$-position and following dehydrogenation, such the migration of methyl groups being considered to occur possibly through the process involving the oxidation of 2- and $2'$-position, and the retropinacolinic rearrangement. However the clear indication on the mode of formation of the pigment can not easily be given from our knowledge of carotenoid biogenesis.

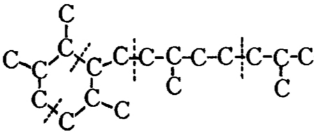


Fig. 4. Isoprene residues in isorenieratene.

Experimental

Isorenieratene prepared in the way described in the preceding papers[1,2] was employed. It formed purplish red needles, m. p. 199°. Absorption maxima were 520, 484, 452 $m\mu$ in carbon disulfide.

Anal. Found: C, 90.93; H, 9.07 %. Calcd. for $C_{40}H_{48}$: C, 90.85; H, 9.15 %.

The infrared spectrum.—It was taken in a potassium bromide pellet, with a Perkin-Elmer 21 double beam instrument equipped with a potassium bromide prism. It is given Fig. 2.

Catalytic hydrogenation (method of Kuhn and Möller[9]).—Catalyst, platinum dioxide; solvent, decalin-acetic acid (1 : 1); test substance, sorbic acid. Found: 15.17, 15.18 moles of hydrogen.

Isopropylidene group (method of Kuhn and Roth[10]).—On ozonolysis isorenieratene (15.706 mg.) gave acetone (0.081 mg.) after subtraction of blanc value. It corresponded to 0.05 mole of acetone per mole of the pigment.

Side-chain methyl group (method of Kuhn and Roth[11]).—Isorenieratene (4.390 mg. and 4.473 mg.) gave volatile acid corresponding to 5.93 cc.

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[7] C. H. Eugster, C. Garbers and P. Karrer, *Helv. Chim. Acta*, **35**, 1179 (1952).

[8] Such phenomena are, for example, reviewed in, W. Klyne, "Progress in Stereochemistry", Butterworths Scientific Publications, London (1954), p. 126.

[9] R. Kuhn and E. E. Möller, *Angew. Chem.*, **47**, 145 (1934).

[10] R. Kuhn and H. Roth, *Ber.*, **65**, 1285 (1932).

[11] R. Kuhn and H. Roth, ibid., **66**, 1274 (1933).

and 6.20 cc. of 0.01 N sodium hydroxide solution, which corresponded to 7.15 and 6.92 moles of acetic acid.

Vitamine A activity.—Isorenieratene was examined for three groups of rats in daily doses of 5γ, 20γ and 40γ respectively. All the rats of these groups died within 38 days after decreases of 28, 26 and 30 g. in body weight (average on the rats of each group). The rats of the group for the positive control for which 7γ of β-carotene were applied in daily doses, gained 33 g. on the average in body weight in 30 days.

***cis-trans* Isomerization**[12].—The solution of isorenieratene (1.379 mg.) in benzene (25 cc.) was isomerized by exposure to scattered light for 30 minutes after the addition of iodine (ca. 1.5 % of the weight of the pigment), and the solution as well as the fresh solution was determined spectrophotoelectrically. The absorption curves are given by Fig. 1, which shows a marked *cis*-peak at 351 mμ in the isomerized solution.

Chromic acid oxidation.—a) *Isorenieral and 2,3,6-trimethylbenzaldehyde.* Isorenieratene (67 mg.) was dissolved in a mixture of benzene (70 cc.) and acetic acid (70 cc.) and each 10 cc. portion of the solution (containing ca. 5 mg. of the pigment) was oxidized with the mixture of the aqueous chromic acid solution (0.1 N, 0.2 cc.) and acetic acid (2 cc.) for ca. 10 seconds under vigorous stirring. The reaction mixture was poured into water (20 cc.) covered with petroleum ether (10 cc.), and washed with water several times. The combined solution was dried with anhydrous sodium sulfate and chromatographed on alumina (developer; benzene). The main zone (brown red) afforded isorenieral (10.5 mg.), after elution and recrystallization of the residue of the eluate from petroleum ether. Isorenieral, m. p. 125~127°, mixed m. p. with the sample obtained from renieratene[4], 125~127°, gave absorption maxima, 486, 454 mμ in petroleum hexane.

Anal. Found: C, 87.48; H, 8.94 %. Calcd. for $C_{30}H_{36}O$: C, 87.33; H, 8.80 %. Phenylhydrazone melted at 206°. One of the upper minor zones afforded a minute quantity of crocetin dialdehyde.

From the faint colored part of the chromatogram, between the zone of isorenieral and that of unchanged isorenieratene, yellow oily residue with characteristic odor was obtained by the evaporation of the eluate. The residue was dissolved in methanol (5 cc.), and treated with an aqueous solution containing semicarbazide hydrochloride (20 mg.) and sodium acetate (30 mg.). After 24 hours the solution was somewhat concentrated and the precipitate was recrystallized from benzene in colorless prisms (4.4 mg.,) m. p. 167°, mixed m. p. with 2, 3, 6-trimethylbenzaldehyde semicarbazone (described later), 167°.

b) *Crocetin dialdehyde.* Isorenieratene (40 mg.) was oxidized with chromic acid (0.1 N, 0.5 cc. per 5 mg. of the pigment). The procedure was the same as above except that the reaction time was for ca. one minute. The main zone of the chromatography of the product was orange red and it afforded crocetin dialdehyde, m. p. 189~190°, (3.1 mg.), mixed m. p. with the sample obtained from renieratene, 189~190°. Absorption maxima 458, 430, 407 mμ (hexane). Crocetin dialdehyde was also obtained by the oxidation of isorenieral with chromic acid.

Oxidation with potassium permanganate.—The solution of isorenieratene (69 mg.) in pure benzene (30 cc.) and the solution of potassium permanganate (930 mg.) and sodium carbonate (930 mg.) in water (35 cc.) were mixed under vigorous stirring to form an emulsion at the room temperature and the stirring was continued for 12 hours. An excess of potassium permanganate was decolorized by the addition of the sodium sulfite solution. The mixture was filtered and the precipitate was repeatedly washed with hot water and benzene. In this way totally 183 mg. of the pigment was oxidized. All the filtrates and the washings were combined, and the aqueous layer was separated from the benzene layer (benzene solution A). The aqueous solution was concentrated under diminished pressure to ca. 10 cc., acidified with 20 % sulfuric acid, and extracted with ether. The ether solution was dried with anhydrous sodium sulfate and concentrated, and the partly crystallized residue was extracted with benzene (5 cc.) by refluxing for 5 minutes (benzene solution B). The residue was dissolved in a small quantity of water, and separated by fractional crystallizations into two parts: difficultly soluble colorless needles (15.5 mg.) which melted at 231° without decomposition, and easily soluble colorless plates, m. p. 99° (6 mg.).

The acid of m. p. 99° melted at 188° when dried at 70° under 18 mmHg for one hour. The mixed melting points with hydrated and anhydrous oxalic acid were 99~100° and 188° respectively.

Anal. Found: C, 19.11; H, 5.05 %. Calcd. for $C_2H_2O_4 \cdot 2H_2O$: C, 19.05; H, 4.80 %.

The acid of m. p. 231° is moderately soluble in hot water and difficultly soluble in benzene and in petroleum benzine.

Anal. Found: C, 61.65; H, 5.29 %. Neutralization equivalent, 97.9. Calcd. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19 %. Neutralization equivalent, 97.1 (dibasic acid).

The dibasic acid (7 mg.) and that recovered from the neutralization experiment (ca. 3 mg.) were heated with potassium permanganate (43 mg.) and sodium carbonate (50 mg.) in water (4 cc.) on a steam bath for 18 hours and then evaporated to ca. 0.5 cc. The mixture was acidified with 20 % sulfuric acid and manganese dioxide was dissolved by the addition of concentrated sodium sulfite solution. The clear solution was then extracted eight times with ether (total 150 cc.) and after drying with anhydrous sodium sulfate, ether was evaporated up. The residue was recrystallized from a small quantity of water

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12) L. Zechmeister and P. Tuzson, ibid., **72**, 1340 (1935); L. Zechmeister and A. Polgár, *J. Am. Chem. Soc.*, **65**, 1522 (1943).

using a little active charcoal in colorless prisms (5.2 mg.), m.p. 215∼238° (decomposition), which was identified to be 1,2,3,4-benzenetetracarboxylic acid by an analysis and a mixed melting point with the authentic sample6).

Anal. Found: C, 47.73; H, 2.64 %. Neutralization equivalent, 61.6. Calcd. for $C_{10}H_6O_8$: C, 47.26; H, 2.38 %. Neutralization equivalent, 63.5.

The benzene solution (B) was concentrated under diminished pressure and the oily residue was extracted with petroleum benzine. Evaporation of the solvent of the extract gave a minute quantity of fine needles which was still contaminated by oily substance (the needles showed a m. p. 65∼72° after drying on a porous plate). It was neutralized with 0.01 N sodium hydroxyde solution (2.642 cc.) by the use of phenolphthalein, extracted with ether to remove phenolphthalein, evaporated to dryness and treated with *p*-phenylazophenacyl bromide13) (8.2 mg.) in ethanol for 30 minutes on a steam bath. After evaporation of the solvent, the residue was once recrystallized from methanol and chromatographed on alumina from the benzene solution. The main orange red zone afforded a minute quantity of red needles, m. p. 163°. A mixed m. p. 163° and a mixed chromatography with the authentic sample of *p*-phenylazophenacyl-2, 3, 6-trimethylbenzoate (Red needles, m. p. 163°. *Anal.* Found: C, 74.61; H, 5.84 %. Calcd. for $C_{24}H_{22}O_3N_2$: C, 74.59; H, 5.73 %. The acid was prepared from pseudocumene-3-magnesium bromide by the introduction of carbon dioxide at −7° in 55 % yield14).) proved their identity.

The benzene solution (A) was evaporated up under reduced pressure and the residue was dissolved in methanol (5 cc.) and treated with the solution of semicarbazide hydrochloride (20 mg.) and sodium acetate (30 mg.) in a little water. The solvent was somewhat concentrated and the precipitate was recrystallized twice from benzene-methanol in colorless prisms, m. p. 149∼154° (13 mg.). As it seemed still impure, it was converted into 2, 4-dinitrophenylhydrazone in ethanol containing a little sulfuric acid by the addition of 2, 4-dinitrophenylhydrazine, and chromatographed on alumina from a benzene solution. The main orange zone gave pure 2, 4-dinitrophenylhydrazone, m. p. 219°, (8.5 mg.), in deep red fine needles, when recrystallized from benzene-methanol. From the mother liquor of recrystallization of the above semicarbazone, 3.2 mg. of the hydrazone was further obtained. The mixed m.p. with 2, 3, 6-trimethylbenzaldehyde-2, 4-dinitrophenylhydrazone was 219°.

Anal. Found: C, 58.83; H, 4.99 %. Calcd. for $C_{16}H_{16}O_4N_4$: C, 58.53; H, 4.91 %.

2,4-Dimethylbenzene-1,3-dicarboxylic acid. —2, 3, 6-Trimethylbenzoic acid (165 mg.), potassium permanganate (590 mg.) and sodium carbonate (590 mg.) in water (25 cc.) were vigorously stirred at an ordinary temperature for 10 hours. The mixture was then filtered, and the filtrate, combined with washings, was concentrated to 5 cc. and acidified with 20 % sulfuric acid. The precipitate and the residue of the ether extract of the mother liquor were combined and recrystallized twice from water in colorless needles of m. p. 231° (90 mg.).

Anal. Found: C, 61.69; H, 5.31 %. Neutralization equivalent, 98.1. Calcd. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19 %. Neutralization equivalent, 97.1. The acid gave 1, 2, 3, 4-benzenetetracarboxylic acid on oxidation with potassium permanganate.

2,3,6-Trimethylbenzaldehyde.—Ethyl orthoformate (12 g.) and the Grignard reagent, prepared from 3-bromopseudocumene (16 g.) and magnesium (2 g.) in ether (80 cc.) was gently boiled for 40 minutes. The ether was then distilled off, and after the violent reaction with foaming [a large flask (500 cc.) was employed to avoid the overflowing] was subsided, which took place when the temperature of the water bath reached to 70∼80°, the mixture was heated on a steam bath for further 20 minutes. The product was then decomposed with dilute sulfuric acid, the oil separated was hydrolysed by boiling with 20 % hydrochloric acid and was treated in methanol (50 cc.) with semicarbazide hydrochloride (12 g.) and sodium acetate (20 g.) in water (20 cc.). The precipitate was filtered, washed successively with petroleum ether and with water, and recrystallized from benzene-methanol in colorless plates (10.5 g.), m. p. 167°.

Anal. Found: C, 64.54; H, 7.51 %. Calcd. for $C_{11}H_{15}ON_3$: C, 64.36; H, 7.37 %.

The free aldehyde was obtained by the steam distillation of the semicarbazone with 20 % sulfuric acid. B. p. 137∼138° (30 mm Hg); 7 g. 2, 4-Dinitrophenylhydrazone, red needles, m. p. 219°.

Anal. Found: C, 58.86; H, 5.07 %. Calcd. for $C_{16}H_{16}O_4N_4$: C, 58.53; H, 4.91 %.

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[13] S. Masuyama, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zassi*), **71**, 402 (1950).

[14] H. A. Smith and J. A. Stanfield, *J. Am. Chem. Soc.*, **71**, 81 (1949).