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Polysaccharides in Lichens and Fungi. III.[1,2] Further Investigation on the Structures and the Antitumor Activity of the Polysaccharides from *Gyrophora esculenta* Miyoshi and *Lasallia papulosa* Llano

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The acyl group attached to the antitumor active polysaccharide (GE-3) isolated from *G. esculenta* has been identified as O-acetyl, and the content was determined to be about 2%. The O-acetyl groups were located in the 3-position of the glucose units by employing the method of Bouveng. The molecular weight of the polysaccharide was calculated to be approximately 20000 by equilibrium ultracentrifugation. The antitumor effects of some derivatives of GE-3 were tested against the implanted sarcoma-180. The results suggested that the O-acetyl groups are concerned in the antitumor activity. The identity of the polysaccharide isolated from *L. papulosa* with GE-3 has been further extended, since the acyl group attached to the former was also proved to be O-acetyl.

Recently we have reported the remarkable antitumor effect of the water soluble polysaccharide preparations from some lichens on the implanted sarcoma-180 in mice.[1,4] The active principles were isolated in pure state from *Gyrophora esculenta* Miyoshi (Iwatake in Japanese) and *Lasallia Papurosa* Llano and shown to be identical each other.[5,6] Methylation, partial acid hydrolysis, and enzymolysis studies elucidated their structures to be essentially linear β-1,6-linked glucans. Accordingly, they were colsely related to pustulan in the molecular structures. Pustulan was first isolated by Drake from *Umbilicaria pustulata* and suggested to be a β-1,6-glucan.[7] Later, more confirmatory evidences for the structure of pustulan have been provided by Lindberg.[8] These antitumor active polysaccharides were, however, differentiated from pustulan by the presence of unidentified acyl groups in their molecules.

The present work includes: a) the identification and determination of the acyl groups, b) location of the acyl groups with respect to the hydroxyl groups of the component glucose residues within the polymer, c) determination of the molecular weight, and e) a study of the effects of the acyl groups on the antitumor activity.[9]

The presence of acyl groups in these polysaccharides has been previously indicated[5,6] by the features of their infrared (IR) spectra and by formation of the deacylated glucans on treatment with 2% sodium carbonate solution. In order to give a further evidence for the

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2) Presented at the 41st General Meeting of the Japanese Biochemical Society, Tokyo, September, 1968. Abstracts: *a*) S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *J. Japan. Biochem. Soc.*, **40**, 543 (1968); *b*) F. Fukuoka, M. Nakanishi, S. Shibata, Y. Nishikawa, T. Takeda, and M. Tanaka, *ibid.*, **40**, 543 (1968).
3) Location: *a*) *Hongo, Bunkyo-ku, Tokyo*; *b*) *Tsukiji, Chuo-ku, Tokyo.*
4) S. Shibata, Y. Nishikawa, M. Tanaka, F. Fukuoka, and M. Nakanishi, *Z. Krebsforsch.*, **71**, 102 (1968).
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9) Partly reported in the preliminary communication.[6]

presence of acyl group, the polysaccharide of *G. esculenta* (designated as GE-3)was dialyzed and the impermeable portion was examined with the IR and nuclear magnetic resonance (NMR) spectra. The IR spectrum was identical with that of original GE-3, giving the absorption bands of ester. As shown in Fig. 1, the NMR spectrum revealed a small signal around δ 2.1 ppm (in D_2O, relative to DSS), suggesting the presence of a small amount

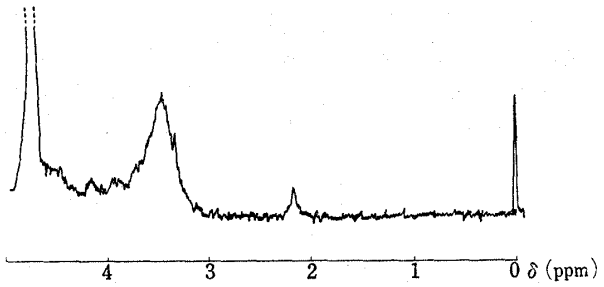

Fig. 1. The NMR Spectrum of GE-3 (in D_2O, DSS as an internal standard)

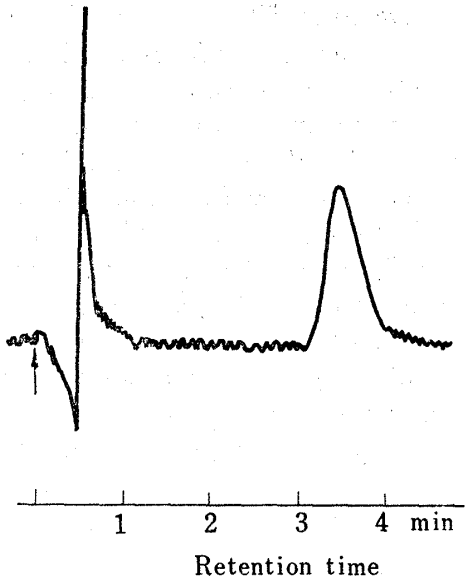

Fig. 2. The Gas Chromatogram of the Acidic Fraction by Saponification of GE-3

of O-acetyl groups in the molecule. To secure the suggestion, the acid liberated after saponification of GE-3 has been identified to be acetic acid by comparison of the *p*-bromophenacyl ester with the corresponding derivative of the authentic sample. (mixed fusion, IR spectra, thin-layer chromatograms and elemental analyses). Furthermore, the acidic fraction separated from saponification was analyzed by gas-liquid chromatography, using Porapak Q column. It gave only one peak, whose relative retention time was exactly equal to that of authentic sample of acetic acid, eliminating the possibility of presence of other acids (Fig. 2). The total acetyl content of the polysaccharide (GE-3) was determined to be approximately 2% by comparing the peak area with those of known amounts of standard acetic acid. The acetyl content corresponded to one acetyl group for every 10 to 12 glucose units. In the similar way, the acyl group of the native polysaccharide (designated as LP-2) obtained from *L. papulosa* was also elucidated to be acetyl. These are the first examples of acetylated polysaccharides isolated from lichens. The presence of O-acetyl groups in many kinds of bacterial polysaccharides has been known in literatures.[10-21] Occurrence of O-acetylated polysaccharides

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has been also reported in higher plants, especially in holocellulose portion of both hardwoods and softwoods.[22-34] For example, O-acetylated glucomannans[23-29] were isolated from pine woods, such as *Pinus silvestris* and *P. densiflora*, and O-acetyl-4-O-methylglucuronoxylans[30-34] from both holocelluloses and sulfite pulps prepared from hardwoods. Among glucans, a few examples are known to occur in nature as acylated forms, *e.g.*, succinoglucan produced by *Alcaligenes faecalis* var. *myxogenes* strain 10C3 with succinyl group[35] and islandic acid and luteic acid obtained from the culture medium of *Penicillium* species with malonyl group.[36]

When the periodate oxidation was carried out with the native (GE-3) and the deacetylated (GE-5) polysaccharides of *G. esculenta*, both samples consumed 2 moles of the reagent per anhydroglucose residue, and no significant difference was observed in their periodate-uptake. In order to obtain a conclusive evidence for locating the O-acetyl groups on the C-3 hydroxyl groups of the glucose units we employed a well-established method. The method used was originally developed by Bouveng[31] to locate the O-acetyl groups in an O-acetyl-4-O-methylglucuronoxylan from white birch. The reaction sequence is shown in Chart 1. The native

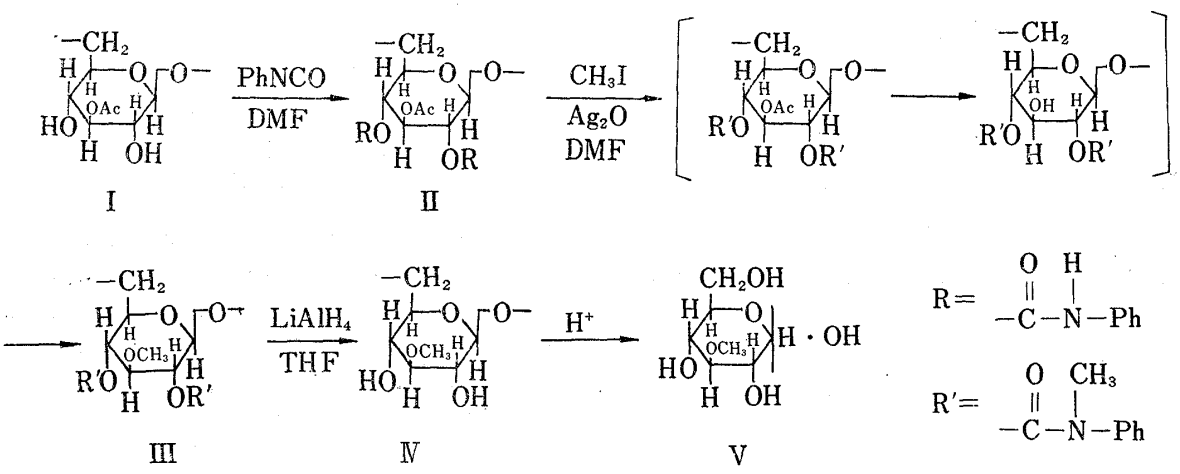

Chart 1

O-acetylated glucan (GE-3) (I) was swollen in dimethylformamide and treated with an excess of phenylisocyanate to convert the free hydroxyl groups to phenylcarbamoyl groups (II). When the phenylcarbamate (II) was methylated by the Kuhn procedure, the O-acetyl groups were replaced by O-methyl groups and the phenylcarbamoyl groups were N-methylated all in one step, to give the O-methylglucan N-methylphenylcarbamate (III). The N-methylphenylcarbamoyl blocking groups were removed by reduction with lithium aluminum hydride

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23) H. Meier, *Acta Chem. Scand.*, **15**, 1381 (1961).
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29) G. Katz, *Tappi*, **48**, 34 (1965).
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in tetrahydrofuran. The distribution of the methoxyl groups in the resulting O-methyl glucan (IV) should have corresponded to the distribution of the O-acetyl groups in the original O-acetylated glucan (I). The O-methyl glucan (IV) was hydrolyzed and the products were analyzed by gas–liquid chromatography and by thin–layer chromatography. Besides D-glucose, a methylated sugar was detected and identified as 3-O-methyl-D-glucose (V), by comparison with the synthetic specimen.[37]

Thus we concluded that the O-acetyl groups were attached to the 3-position of the glucose units within the native polysaccharide (GE-3). Some investigation is now under progress to reveal the distribution of the acetylated glucose units in the whole molecule.

Hitherto, the molecular weight of pustulan from *U. pustulata* has not been reported. The molecular weight of GE-3 has now been calculated to be approximately 20000 by the method of equilibrium sedimentation, using the equation proposed by Yphantis.[38] Although the molecular weights of LP-2 and pustulan (kindly supplied by Prof. B. Lindberg) were not determined, they would have similar molecular-sizes to that of GE-3, since their physical properties were, as shown in the previous paper,[5] closely similar to that of the latter.

As a conclusion, the present data indicated that the antitumor active polysaccharide of *G. esculenta* was composed of approximately 120 glucose units and about 10% of them carried O-acetyl groups in the 3-position.

The antitumor effects of four kinds of derivatives of GE-3, *e.g.*, the deacetylated glucan (GE-5), peracetate, permethylate, and phenylcarbamate, were tested against implanted sarcoma-180 in mice under the same conditions as described in our previous paper.[1] Preparation and characterization of the first three samples have already been reported.[5] The results obtained are summarized in Table 1. The deacetylated glucan (GE-5) was found to be hi-

Table I. Antitumor Effect[a] of Some Derivatives of the Polysaccharide (GE-3) Isolated from *G. esculenta*

Samples	Dose (mg/kg × day)	Inhibition ratio (%)	Complete regression	Mortality (died/total) Control	Mortality (died/total) Treated	Average body wt. change (g) Control	Average body wt. change (g) Treated
Deacetylated glucan (GE-5)	150×10	85.5	1/8	0/8	0/8	+7.5	+6.6
	150×8	78.0	0/7	2/9	3/10	+4.6	+5.0
	100×1						
GE-3 acetate	150×10	9.3	0/10	0/10	0/10	+1.8	−0.8
	150×10	4.9	0/9	0/8	0/9	+3.0	+2.7
GE-3 methylate[b]	150×10	−2.0	0/10	0/10	0/10	+1.5	+3.5
GE-3 carbamate	150×10	−25.7	1/9	0/10	1/9	+5.2	+5.0
cf[c]							
Native glucan (GE-3)	200×10	99.1	8/10	1/10	0/10	+3.0	+5.8
	150×10	97.3	7/10	0/10	0/10	—	—
	100×10	91.3	4/10	0/10	0/10	+2.8	+3.4

a) tumor, sarcoma-180 (solid); animal, swiss albino mouse; route, *i.p.*; vehicle, aq. dest.
b) In this sample, the original O-acetyl groups were replaced by O-methyl groups.
c) For comparison, data were cited from our previous paper.[1]

ghly effective, but its inhibition ratios were, though slightly, lower than those of the original polysaccharide (GE-3). A more significant difference between their effects could be observed in the number of mice where complete regression of the tumor took place. On the other hand, peracetylation of GE-3 caused a complete loss of the effect. The results are of indicative that the presence of O-acetyl groups in the molecule is not essential for appearance of the

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38) *a*) D.A. Yphantis, *Ann. New York Acad. Sci.*, **88**, 586 (1960); *b*) *Idem*, *Biochemistry*, **3**, 297 (1964).

effect, but the difference of acetyl content is accompanied by changes in the antitumor activity. Other samples, methylate and phenylcarbamate, were also shown to be ineffective, implying that complete blocking of the free hydroxyls by substitution would cause a total loss of the effect.

Although we are not in a position to discuss the reason of the present results, it may be noteworthy to mention that a number of type-specific polysaccharides from Pneumococci, *e.g.*, S. 1,[12] S. 11A,[13] S. 18,[14] and S. 34,[10,11] contain O-acetyl groups in their molecules which reveal the important relation with their immunological specificity. Katz reported the significant effect of the O-acetyl groups on the physical properties of the O-acetyl glucomannan from Parana Pine.[29] His results indicated that the O-acetyl groups were effective in preventing molecular orientation and subsequent development of lateral order in the polysaccharide. Such effect of the O-acetyl groups should not be lost sight of.

Experimental

The infrared spectra were measured with Japan Spectroscopic Co. Model DS-402G Spectrophotometer, and the nuclear magnetic resonance spectrum with a Japan Electron Optics Lab. JMN-3H-60 spectrometer. Gas-liquid chromatographic analyses were carried out with a Shimazu Model GC-1B Gas Chromatograph attached with a hydrogen flame detecter. The ultraviolet absorbances were measured with a Cary self-recording spectrophotometer Model 11. A Spinco Model E analytical ultracentrifuge with a schlieren optical system was used for determination of molecular weight.

Materials——Preparation and characterization of the samples, GE-3 (native polysaccharide of *G. esculenta*), GE-5 (deacylated polysaccharide derived from GE-3), peracetate of GE-3, permethylate of GE-3, and LP-2 (native polysaccharide of *L. papulosa*) have been reported in our previous papers.[1,5,6] In these cases, the same batches of the samples were used in the following experiments.

Identification of O-acetyl groups in the Molecules of GE-3 and LP-2——i) Dialysis of GE-3: An aqueous solution of GE-3 was dialyzed with Visking tube against running water for 3 days and then against distilled water for several hours. To the impermeable solution EtOH was added to form a precipitate, which was collected by filtration, washed with ether and acetone, and then dried. The infrared spectrum of the recovered polysaccharide was identical with that of the original GE-3, showing the absorption bands of ester. IR ν_{max}^{KBr} cm^{-1}: 1735, 1250 (ester), 910 (β-linkage). The nuclear magnetic resonance spectrum was measured at 60 Mc in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. As shown in Fig. 1, a signal (broad) was observed around δ 2.1 ppm, suggesting the presence of acetyl groups in a small portion.

ii) Preparation of Acidic Fraction by Saponification: GE-3 (10.0 g) was suspended in 0.2% NaOH (300 ml), and the mixture was stirred for 30 min at room temperature. An acidic supernatant was separated from the precipitated material (GE-5) by centrifugation.

iii) Preparation of *p*-Bromophenacyl Ester: A part of the supernatant was neutralized with $NaHCO_3$, and then evaporated to dryness *in vacuo*. The residue (25.3 mg) was dissolved in H_2O (6 drops) and added with 0.5N HCl solution (22 drops). To the solution, *p*-bromophenacyl bromide (86 mg) in EtOH (1 ml) was added and the whole mixture was refluxed for 1 hr. After cooling in an ice-bath, a precipitate formed was collected by filtration. The product was chromatographed on Silicagel G with $CHCl_3$ to remove the excessive reagent and then recrystallized from aq. EtOH to give colorless leaflets. The *p*-bromophenacyl ester had a mp and mixed mp of 85—85.5° with authentic *p*-bromophenacyl acetate. The identity was also proved by comparison of their infrared spectra and thin-layer chromatograms on Silica gel (Camag D-5) with $CHCl_3$. *Anal.* Calcd. for $C_{10}H_9O_3Br$: C, 46.69; H, 3.50. Found: C, 46.71; H, 3.78.

iv) Gas-Liquid Chromatography: A part of the acidic supernatant was passed through the column of Dowex 50W-X8 (H^+ form), and then analyzed by gas-liquid chromatography, using Porapak Q column[39]; column temperature, 200°, detecter temperature, 220°, carrier gas, N_2. As shown in Fig. 2, it gave only one peak, whose relative retention time (3.4 min) was equal to that of authentic acetic acid. The acetyl content of GE-3 has been determined to be approximately 2% by gravimetrical comparison of the peak area of the sample with those of known amounts of authentic acetic acid.

v) Evidence for Acetylation of LP-2: The acid liberated by saponification was proved to be acetic acid by gas-liquid chromatographic analyses and by preparation of the *p*-bromophenacyl ester in the similar manner as described in the case of GE-3.

39) A product of Waters Associates Inc.

Determination of Molecular Weights of GE-3 and GE-5——This was made by the method of sedimentation equiliblium, using the equation proposed by Yphantis.[38] Distilled water was used as a solvent. A partial specific volume of 0.612 per g was estimated by picnometer. Based on the value the molecular weight of GE-3 was calculated to be approximately 20000 (DP *ca.* 120). The molecular weight of GE-5 was also estimated in a similar way. The value obtained was nearly equal with that of GE-3.

Periodate Oxidation of GE-3 and GE-5——GE-3 (49.25 mg) was dissolved in H_2O (10 ml), $NaIO_4$ (0.01M, 20 ml) was added, and the solution was stored at room temperature in the dark. A blank experiment was done under the same conditions. Aliquots of the sample and the blank were removed at intervals and the absorbances of the resulting solutions were measured in the spectrophotometer at 223 mμ and compared with those of the original solution of periodate and of an equimolar iodate solution. The periodate-uptake of GE-5 was estimated under the similar conditions. The number of moles of $NaIO_4$ consumed per anhydroglucose unit of polysaccharide were as follows (moles/mole (min)): GE-3; 0.93 (30), 1.42 (60), 1.75 (120), 1.86 (150), 1.96 (180), 1.98 (210), 2.01 (240), 2.01 (1230). GE-5; 1.38 (30), 1.98 (90), 1.98 (150), 2.02 (210), 2.04 (270), 2.01 (1230).

Preparation of Phenylcarbamate of GE-3——Dry GE-3 (5.6 g) was swollen in 110 ml of dry dimethylformamide and then 15 ml of phenylisocyanate were added. After refluxing on a water-bath for 14 hr, the reaction mixture was cooled and the precipitate formed was collected by centrifugation. The supernatant was poured into EtOH and the resulting precipitate was collected by filtration. The precipitates were combined together, washed with EtOH and ether, and dried. Two more repetition of the reaction yielded a slightly greyish white powder (II) (12.5 g), whose IR spectrum was characteristic of a mono-substituted urethane. IR ν_{max}^{KBr} cm^{-1}: 3280 (N-H stretch), 1730—1720 (ester-amide carbonyl and acetyl carbonyl), 1603 (phenyl) and 980 (β-glucosidic linkage). IR ν_{max}^{Nujol} cm^{-1}: 3230 (N-H stretch), 1735 (acetyl), 1700 (ester-amide), 1600 (phenyl), 890 (β-glucosidic linkage). The antitumor effect is shown in Table I.

Methylation of GE-3 Phenylcarbamate——The phenylcarbamate (II) (5 g) was dissolved in dimethylformamide (100 ml), and CH_3I (19 ml) and freshly prepared Ag_2O (17 g) were added. The whole mixture was refluxed for 20 hr under stirring. After filtration, EtOH was added to the filtrate to give a precipitate, which was collected by centrifugation, washed with EtOH and ether, and then dried. The process was repeated twice to yield 7.4 g of N-methylphenylcarbamate (III). IR ν_{max}^{Nujol} cm^{-1}: 1710 (ester-amide carbonyl), 1603 (phenyl), 890 (β-glucosidic linkage). The absence of O-acetyl groups in the methylation product was verified in the following manner. A portion of the product (500 mg) was dissolved in tetrahydrofuran (8.7 ml) and 33% H_2SO_4 (0.56 ml) was added. After standing at room temperature for seven days, EtOH was added and the precipitate was washed and dried in the usual manner. The IR spectrum of the acid-treated methylation product showed no hydroxyl bands. The IR spectra of the methylation product before and after the acid treatment were identical. The result indicated that the replacement of the O-acetyl groups by methyl groups was complete.

Lithium Aluminum Hydride Reduction of the N-Methyl-phenylcarbamate——The N-methylphenylcarbamate (III) (5 g) was dissolved in dry tetrahydrofuran (200 ml) and powdered $LiAlH_4$ (1.5 g) was added. The solution was stirred at room temperature for 20 min and then refluxed for 1 hr with continued stirring. After cooling, an additional $LiAlH_4$ (1.75 g) was added and the mixture was stirred continuously for 20 hr. The excess $LiAlH_4$ was destroyed by the dropwise addition of distilled H_2O over a period of a few hr. The mixture was acidified to pH 6 with 5% H_3PO_4 and allowed to stand overnight. The precipitated materials were collected by filtration and dried to afford 2.5 g of slightly greyish white powder. The absence of aromatic bands in the IR spectrum of the product indicated that the reduction was complete and the formation of O-methyl glucan (IV). IR ν_{max}^{Nujol} cm^{-1}: 3460 (O-H), 890 (β-linkage).

Hydrolysis of the O-Methyl Glucan——The O-methyl glucan (IV) (2 g) was treated at room temperature for 1 hr with 72% H_2SO_4 (40 ml). After addition of 160 ml of H_2O, the solution was heated on a water-bath for 5 hr. The hydrolyzate was cooled and neutralized with saturated Ba $(OH)_2$ solution. The mixture was centrifuged and the salts washed twice with 50% aqueous EtOH. Concentration of the supernatant liquid afforded a syrup, which was subjected to the thin-layer and gas-liquid chromatographic analyses. Thin-layer chromatography was carried out on Avicel (the Avicel Sales Division of American Viscose Co.), using a solvent system of AcOEt-HCOOH-H_2O (6:3:2). The *Rf* values (0.52 and 0.74) of the two spots detected with anilinhydrogenphthalate reagent were identical with those of authentic D-glucose and the synthesized 3-O-methyl-D-glucose,[37] respectively. Gas-liquid chromatography was performed under the following conditions: column, SE-52; column temperature, 180°; detector temperature, 230°; carrier gas, N_2: flow rate, 30 ml/min; column length, 2 m. Retention times (min): Sample; 1.8, 3.3, 6.0, and 8.8. D-Glucose; 6.0 and 8.8. Synthesized 3-O-methyl-D-glucose[37]; 1.8 and 3.3.

Assay Method of Antitumor Effect——The test was made by observing the effect on the growth of subcutaneously implanted sarcoma-180 (solid) for 5 weeks. Details of the method were described in our previous paper.[1] The results are shown in Table I.

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