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*Communication*

# Complete Assignment of the $^{1}H$ and $^{13}C$ NMR Spectra of Carthamin Potassium Salt Isolated from *Carthamus Tinctorius* L.

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**Abstract:** Carthamin potassium salt isolated from *Carthamus tinctorius* L. was purified by an improved traditional Japanese method, without using column chromatography. The $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) signals of the pure product were fully assigned using one- and two-dimensional NMR spectroscopy, while the high purity of the potassium salt and deprotonation at the 3′ position of carthamin were confirmed by atomic adsorption spectroscopy and nano-electrospray ionization mass spectrometry.

**Keywords:** carthamin-3′potassium salt, green metallic luster, fermented safflower petal tablet

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Carthamin, a traditional red pigment obtained from the dried petals of safflower (*Carthamus tinctorius* L.), has long been used in food colorants, dyes, and medicines worldwide. The mostly yellow appearance of the lively safflower petals reflects the prevalence of water-soluble yellow ingredients. Originally native to Asia Minor, safflower spread to central Europe (via Egypt) and Japan (via China) [1]. In Japan, a safflower-derived red pigment known as "beni" [2] was commonly traded and used in cosmetics despite being expensive and rare (Figure 1).

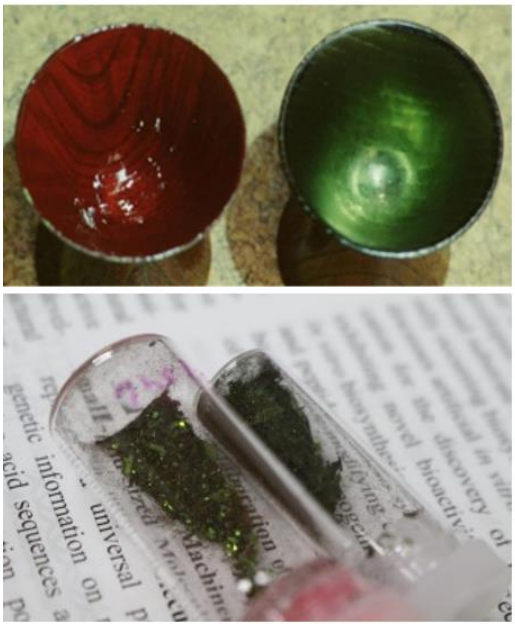

**Figure 1.** Beni, a typical Japanese cosmetic, in wet (left) and dry (right) states (top). Dried stable beni isolated and purified in this work (bottom).

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In particular, the green luster of beni was viewed as evidence of its high quality, and the corresponding pigment was called "sasairo-beni" (bamboo-colored red). Today, we know that safflower petals contain yellow and red pigments [3], with the red pigment (represented by a single compound) accounting for <1% of the total pigment content. This rare red pigment, viz. carthamin, was first reported in 1846 [4]. Since then, carthamin purification methods and structure have been extensively investigated [5-9] and the correct molecular structure (C-glycoside with two glucose residues) was determined in 1979 [10,11]. Subsequently, the total synthesis of carthamin was achieved [12,13] and a hybrid bio-/organic synthesis incorporating enzymatic reactions was then proposed [14]. These syntheses confirmed the skeletal and full molecular structures of carthamin (Figure 2).

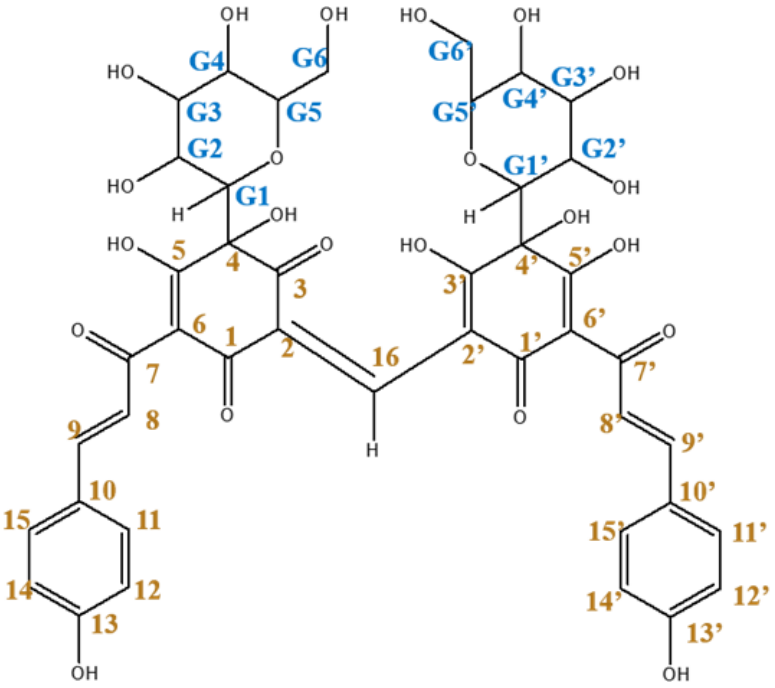


**Figure 2.** Molecular structure of carthamin

Typically, the isolation of carthamin from safflower petals and its purification are performed as follows. Dry safflower petals are suspended in cold water, allowed to stand for some days to remove the yellow pigments, and repeatedly washed with running water until the disappearance of the yellow color. The petals in the filter bag are then transferred to a new vessel filled with fresh cold water containing sodium bicarbonate, and the filter bag is kneaded to release the red pigment into the solution. However, the purity of the thus obtained product is insufficient for structure elucidation, which has inspired numerous attempts to (i) form pure crystals using various derivatizations and treatments as well as (ii) achieve separation using column chromatography. To date, complete assignments of the $^{1}H/^{13}C$ nuclear magnetic resonance (NMR) and mass spectra of carthamin have not been reported [13,15-18], which can be ascribed to the light- and temperature-sensitive nature of this pigment and the problems associated with its isolation.

Our research initially focused on the mechanism of "bamboo color" development, as the origin of carthamin's green color (which is actually not a structural color but a metallic luster) [19-21] has not been investigated. Therefore, a complete assignment of all $^{1}H$ and $^{13}C$ signals in the NMR and mass spectra of carthamin was required to bring its structural analysis to the next stage. Previously, potassium and pyridinium salts of carthamin have been reported through its purification and determination. To realize full NMR and mass-spectral analyses, we used the potassium salt of carthamin, which has a green metallic luster. This purified salt was obtained from benimoti (fermented safflower

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petal tablet: Yamagata Prefecture, Red Flower Production Association) using a traditional Japanese purification method. Specifically, a muddy red precipitate was produced from fermented dried safflower petal tablets (100 g) through more than 12 steps using natural traditional acidic and alkaline solutions and ramie fibers. The precipitate was spread on glass to block light, air-dried at room temperature, and vacuum-dried for five days to reproducibly obtain a red pigment with a green metallic luster (258 mg) [19,20]. Only one red spot with $R_f$ = 0.42 was observed by TLC (eluent = 1-butanol:acetic acid:water, 4:1:5, v/v/v). Nano-electrospray ionization mass spectrometry (NanoESI-MS): *m/z* Found 987.1343, Calcd. 987.9698 for $C_{43}H_{41}O_{22}K_2$; K;10.1 wt%, Na; 0.038 wt%. The NanoESI-MS spectrum showed no peaks assignable to free carthamin. Moreover, the potassium salt was relatively stable, and the NMR solution samples in dimethyl sulfoxide (DMSO-$d_6$) or pyridine-$d_5$ did not change after several years at r.t. Herein, we aimed to assign all the signals in the $^1$H and $^{13}$C NMR spectra of carthamin potassium salt solutions in DMSO-$d_6$.

The assigned NMR spectra were almost identical to the unassigned spectra reported previously [11,14,17] In previous studies, the NMR spectra of carthamin were recorded in a mixture of pyridine-$d_5$ and methanol-$d_4$ [18]. The very broad OH signals observed in pyridine [13] collapsed into a single peak upon the addition of methanol, which did not allow one to extract any information pertaining to the OH groups. Figures 3 and 4 present the assigned $^1$H and $^{13}$C NMR spectra of carthamin potassium salt in DMSO-$d_6$, respectively. Diffusion-ordered spectroscopy (DOSY) spectra revealed that signals below 2.8 ppm were observed on different diffusion lines and did not originate from carthamin. Therefore, these signals were ascribed to a trace impurity not detectable by TLC or NanoESI-MS.

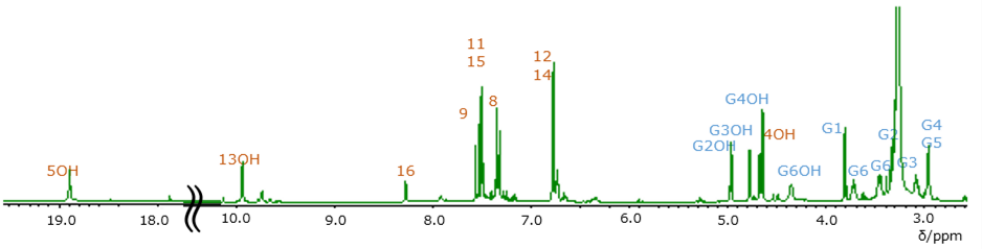


**Figure 3.** Assigned $^1$H NMR spectrum of carthamin potassium salt recorded in DMSO-$d_6$ at 30 °C.

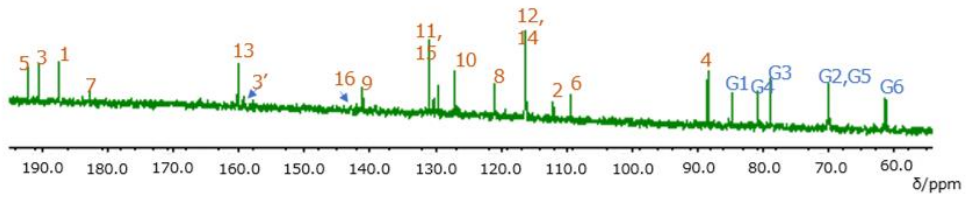


**Figure 4.** Assigned $^{13}$C NMR spectrum of carthamin potassium salt recorded in DMSO-$d_6$ at 30 °C.

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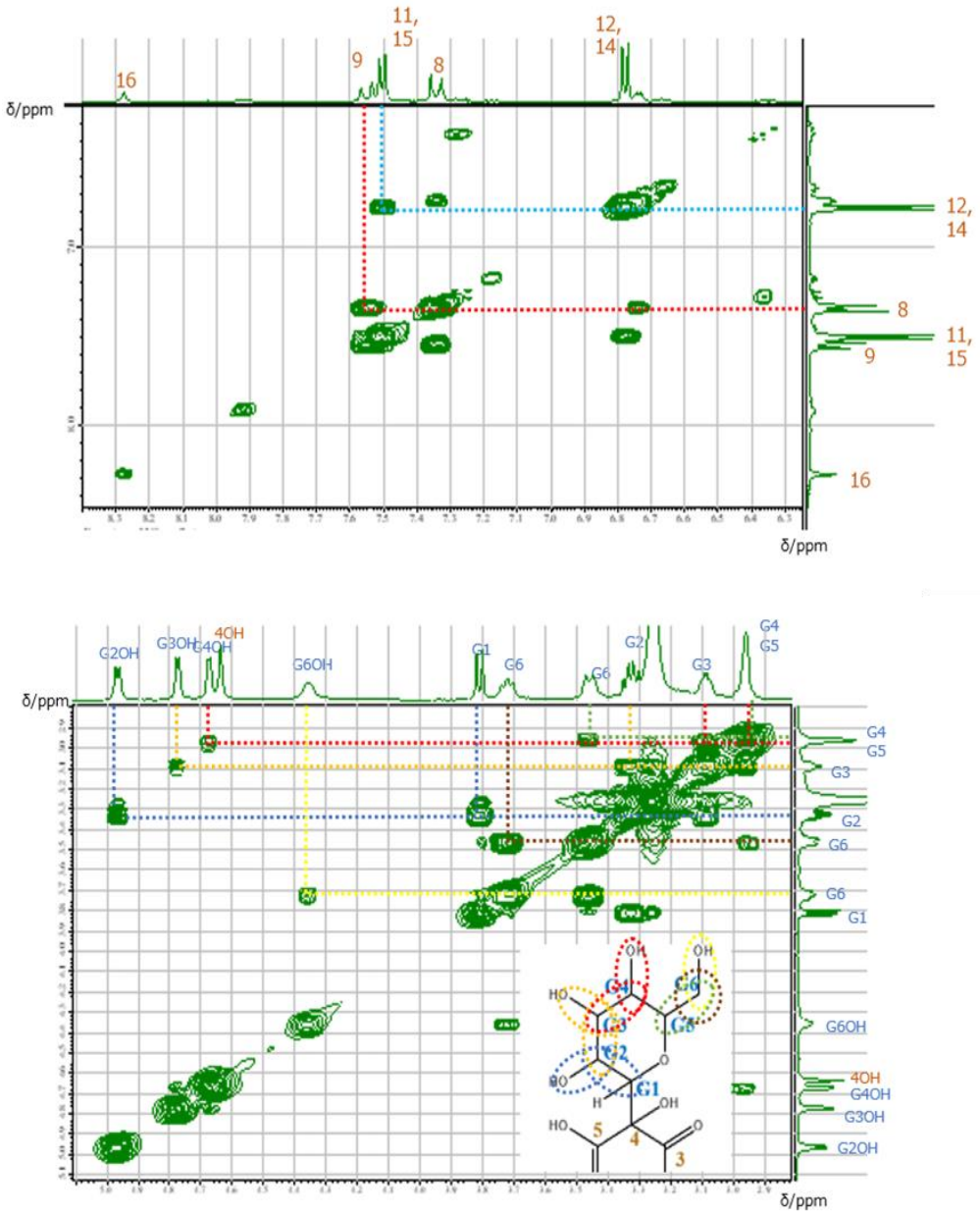


**Figure 5.** COSY spectra of carthamin potassium salt recorded in DMSO-$d_6$ at 30 °C; cross-peaks between carbon atoms (top) and between carbon and oxygen atoms (bottom).

The carthamin protons resonated at 2.97, 3.09, 3.33, 3.46, 3.72, 3.81, 4.36, 4.64, 4.67, 4.77, 4.97, 6.87, 7.34, 7.51, 7.55, 8.28, 9.90, and 18.89 ppm. The signals at 4.36, 4.64, 4.67, 4.77, 4.97, 9.90, and 18.89 ppm disappeared after the addition of $D_2O$ and were therefore assigned to OH groups. The signal at 18.89 ppm has previously been ascribed to the enolic proton of the yellow pigment safflomin A, ((4S)-4,6-di-D-glucopyranosyl-4,5 -dihydroxy-2-[*E*-1-hydroxy-3-(4-hydroxyphenyl)prop-2-enylidene]-cyclohex-5-ene-1,3-di on) from *Carthamus tinctorius* L. [23,24], with the remarkable low-field shift attributed to hydrogen bonding between C–OH and C=O. In our case, the signal at 18.89 ppm was assigned to the 5 and 5′ enolic protons of carthamin. As the signal of the phenolic OH groups of safflomin A at 9.79 ppm was broad, the broad signal at 9.90 ppm was assigned to the 13 and 13′ phenolic OH groups in carthamin. The integrated signal intensity ratio

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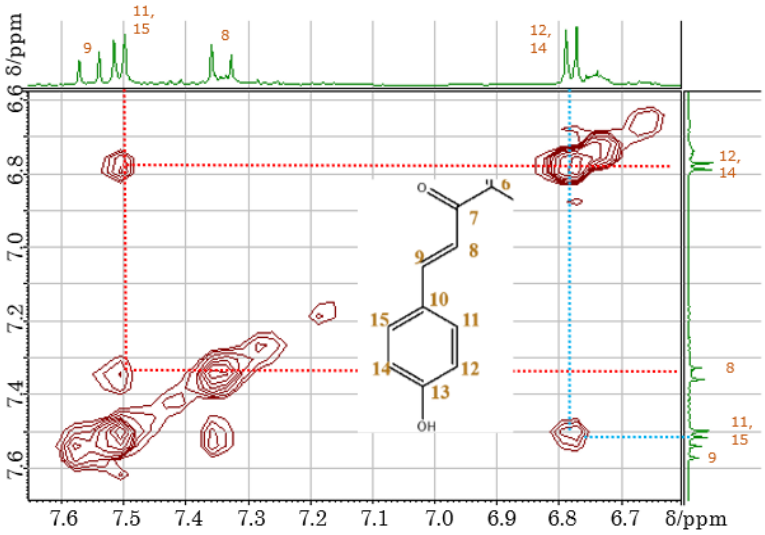


**Figure 6.** NOESY spectrum of carthamin potassium salt recorded in DMSO-$d_6$ at 30 °C.

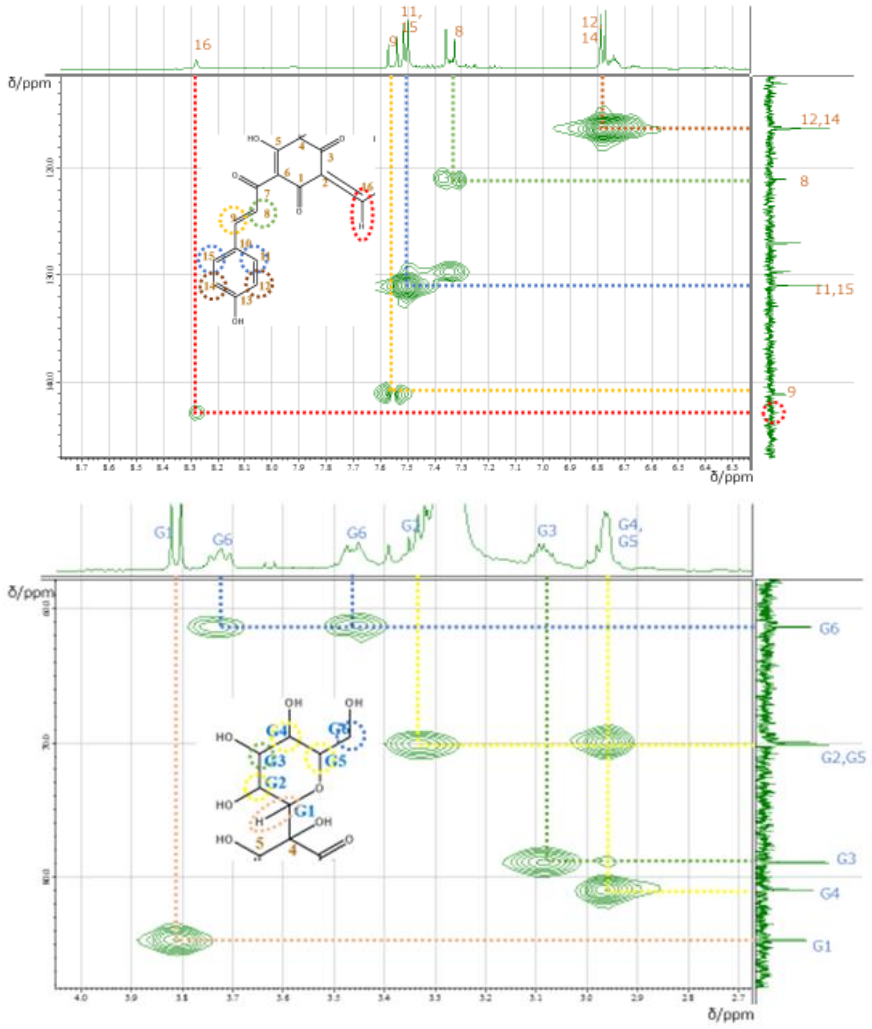


Figure 7. HMQC spectra of carthamin 3′potassium salt recorded in DMSO-$d_6$ at 30 °C under low (top) and high (bottom) magnetic fields.

was 1 (8.28 ppm):2 (3.09, 3.33, 3.46, 3.72, 3.81, 4.36, 4.64, 4.67, 4.77, 4.97, 7.34, 7.55, 9.90, and 18.89 ppm):4 (2.97, 6.87, and 7.51 ppm). The signal at 8.28 ppm was assigned to 16H.

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Correlation spectroscopy (COSY) revealed that cross-peaks were present not only between the signals of protons bonded to adjacent carbons, but also between the signals of protons bonded to adjacent carbon and oxygen atoms[21], which allowed us to assign numerous couples (Figure 5).

The signals of 11 and 11′ overlapped with those of 15 and 15′, while the signals of 12 and12′ overlapped with those of 14 and 14′. Furthermore, nuclear Overhauser effect spectroscopy (NOESY) revealed the presence of cross-peaks between signals at 7.34 and 7.51 ppm, which allowed the signals at 7.34, 7.55, 6.87, and 7.51 ppm to be ascribed to 8H, 9H, 12H overlapped 14H, and 11H overlapped 15H, respectively (Figure 6). According to the molecular structure model, the cross peak was between 8H and 11H or 15H. Thus, only the signal of the 3′OH proton remained unassigned.

Among the 43 carbons constituting carthamin (and affording 23 signals), six glucose-derived carbons were in almost identical environments and therefore featured the same

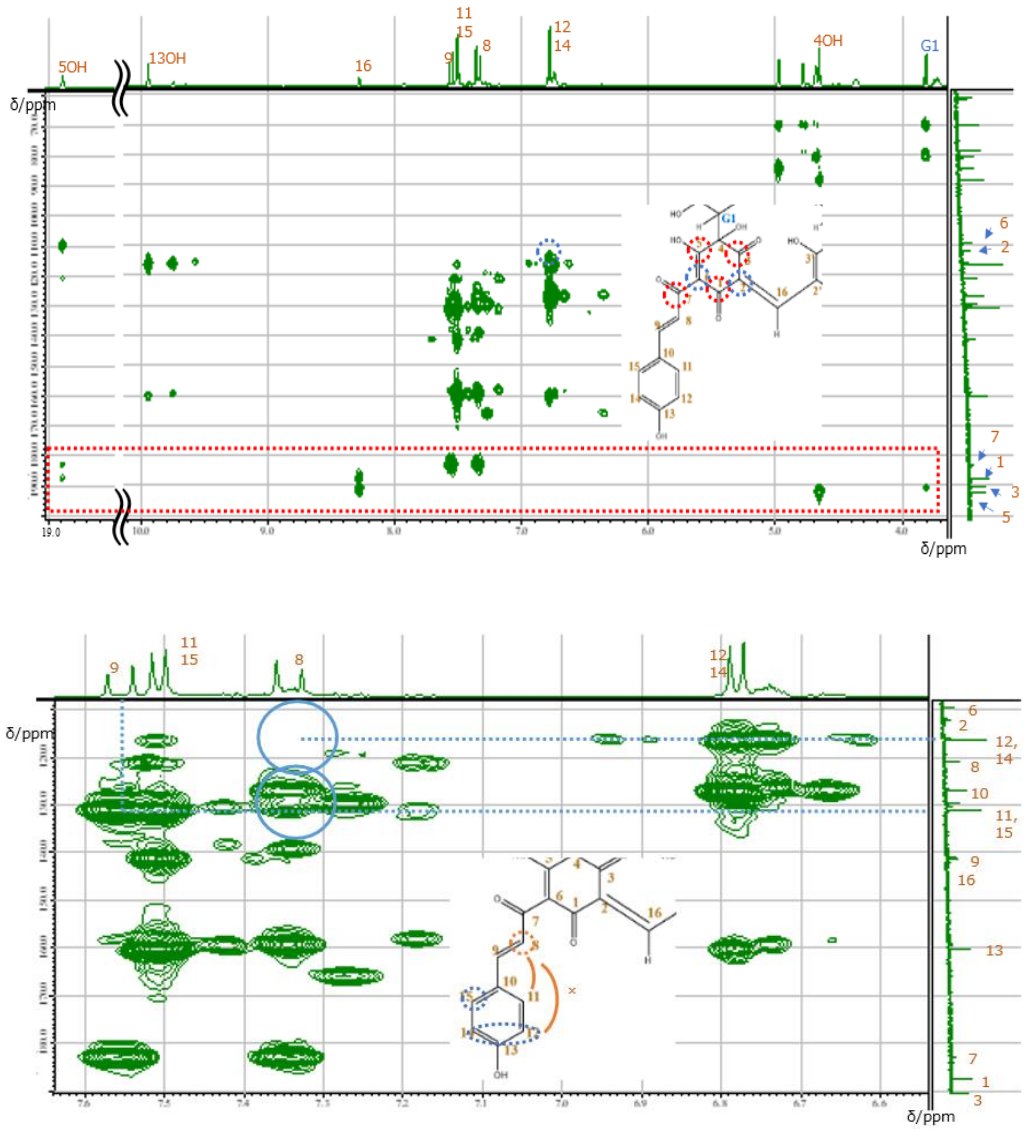


**Figure 8.** Full-range (top) and expanded (bottom) HMBC spectra of carthamin potassium salt recorded in DMSO-$d_6$ at 30 °C.

shift, as did another group of 14 carbons (1, 2, 4–15). The cross-peaks revealed by heteronuclear multiple quantum correlation spectroscopy (HMQC) (Figure 7) allowed us to assign proton-bearing carbons on the glucose ring (G1, G2, G3, G4, G5, and G6) as well as carbons 8, 9, 11, 12, 14, 15, and 16, whereas the long-range correlation data provided by heteronuclear multiple-bond correlation spectra. Protons through oxygen elements

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bonding carbons on the glucose ring (G1, G2, G3, G4, G5, and G6) as well as carbons 8, 9, 11, 12, 14, 15, and 16, whereas the long-range correlation data provided by heteronuclear multiple-bond correlation spectroscopy (HMBC) allowed us to assign carbons 1, 2, 3, 4, 5, 6, 7, 10, and 13 (Figure 8).

The carbonyl (3) and enolic (3') carbons were observed as two separate signals, whereas carbon 16 yielded one signal. Except for the signal derived from 3'C at 159.3 ppm, the above data agree with those reported previously in works not attempting to perform spectral assignments.

To obtain information on the content of inorganic elements and completely assign the $^{1}H$ and $^{13}C$ NMR signals of carthamin, we scrutinized the traditional methods and carried out atomic adsorption and NanoESI-MS analyses. Molecular structure analyses, including solid-state structure analysis, are currently underway. The presented information shows that carthamin is no longer "difficult to analyze" and contributes to safflower petal research and quality control.

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The NMR spectra were recorded on a JEOL JNM-ECZ500R (JEOL Ltd.) spectrometer at 30 °C. DMSO-$d_6$, pyridine-$d_5$, and $D_2O$ were purchased from Kanto Kagaku Co. NanoESI-MS analysis was performed on a QExactive Plus (Thermo Fisher Scientific) instrument, and potassium content was determined using atomic absorption spectrophotometry (Z-2300, Hitachi High-Tech Science Co.). Mass spectral and elemental analyses were performed at Toray Research Centre, Inc.

**Author Contributions:** For research articles with two authors, a short paragraph specifying their individual contributions must be provided. Investigation of NMR data curation was carried out by M. Sasaki and K. Takahashi. Original draft preparation was carried out by Keiko Takahashi, review and editing were carried out by both M. Sasaki and K. Takahashi. Project administration was taken by K. Takahashi. All authors have read and agreed to the published version of the manuscript. Authorship must be limited to those who have contributed substantially to the work reported.

**Acknowledgments:** This research was supported by a Grant-in-Aid for Scientific Research (20K01108) and by the International Center for Science and Arts of Color, Tokyo Polytechnic University in 2020.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds, fermented safflower petal tablet are available from the authors. Purified carthamin-3' potassium salt, if it is mg order, are available from the authors.

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